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RESPONSE OF TESTES OF IMMATURE PIGEONS TO GONADOTROPHINS

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THE prompt and pronounced response of immature avian testes to injections of hypophyseal gonadotrophic hormone is well established (1–7). Current use of weight of the bird testis in biological assays of gonadotrophin requires definite information concerning the nature and sequence of the changes which occur within testes subjected to such stimulation. Further information is needed also on differences of response to two or more types of gonadotrophins. It is now evident that two gonadotrophic principles (follicle stimulating, FSH; and luteinizing, LH) affect different tissues of the mammalian ovary (8, 9). In the rat testis it seems that only germinal elements are stimulated by FSH and only interstitial cells are stimulated by LH (10).

Effects of the two types of gonadotrophins on special tissues of the gonads of birds is not satisfactorily known. Riddle (2, 3) reported that 10 daily injections of an unfractionated extract and suspension of beef anterior lobe may increase the weight of the Ring dove testis by 1,500 to 2,000% and stated that "this phenomenal increase in weight is characterized by a true growth and differentiation of testis tubules already present." Photomicrographs supported that conclusion. Greep (11) injected pigeons 4 or 5 weeks old with preparations especially made to effect a separation of FSH and LH. After 7 days of treatment with such FSH the testes showed marked proliferation of the germinal epithelium; it was stated that sufficiently large (otherwise undefined) dosage of an LH preparation gave a most exaggerated development of interstitial tissue but no statement was made concerning an effect or lack of effect of LH on the development of tubules. In mature hypophysectomized male rats Greep and Fevold (12) later confirmed the above noted response to FSH; they further reported that, in addition to maintaining interstitials and secondary sexual organs, LH also sustained or initiated spermatogenesis. Breneman (13, 14) obtained marked growth of the seminiferous tubules following injections of similar FSH preparations into chicks aged 5 to 15 days. Hypophyseal LH produced testes only slightly heavier than those of controls, and though tubule diameters were not enlarged a definite increase in interstitial tissue was reported. Breneman also made tests of the action of pregnancy urine and mare serum on the chick testis which will be discussed later.

In a preliminary report (15) on a part only of the present material we stated that hypophyseal and certain other gonadotrophic preparations injected into 2-month-old pigeons or Ring doves promptly stimulated their dormant testes, enlarging seminiferous tubules at 4 days and leading to sperm production after 10 to 14 days. In addition

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to the tubular development observed in Ring doves at 4 days, when their testes were increased by 500 to 600% over their original weight, large fluid filled intertubular spaces were present; within these spaces, however, little or no increase in intertubular tissue had occurred. Similar intertubular spaces were not found at any stage of stimulation of testes of common pigeons, although some hypertrophy and also a less dense condition of the intertubular tissue was observed in the testes of the injected birds. In both the dove and pigeon, following a uniform and fairly high level of dosage for 7 to 10 days, these intertubular spaces or tissues became much less evident and the pronounced development of tubules earlier reported by Riddle (2) were regularly obtained.

METHODS AND MATERIALS

This report is concerned chiefly with the growth of tubular and intertubular elements in the stimulated testes of pigeons, and with the changing relative proportion of these elements during the normal growth of the pigeon testis. The presence of fluid filled interstices in the early stages of stimulation in dove testes and their absence in the testes of pigeons indicated that the latter species would be more suitable for that part of our study in which estimates of intertubular tissue were to be made.

Twenty-eight White Carneau pigeons at 1.5 months after hatching were injected daily with 10 mg. of preparation 730 for periods of 4, 8, 12 or 16 days. No. 730 is a preparation [so-called, copper-soluble, of Bates and Riddle (16)] from beef pituitaries which strongly stimulated the ovaries of adult hypophysectomized pigeons. In terms of the chick units defined by Bates, Riddle and Miller (17) 1 mg. of 730 contained 0.8 U gonadotrophin, 1.2 U thyrotrophin, 0.124 U adrenotrophin and less than 0.002 dove units of prolactin. Regular contamination, particularly with thyrotrophin, has led us to designate all such preparations as "FSH+." No. 730 had little or no ability to stimulate the seminal vesicles of 21-day rats but it strongly stimulated the immature rat ovary and uterus. Its high content of glucosamine (8.5%) indicates an FSH+ preparation fairly free from LH (18).

The birds were killed 24 hours after the last of their series of injections and their testes carefully removed, weighed and fixed in Bouin's solution. The tissues were cut at 6 and 10μ and stained with Mallory's triple stain or hematin-eosin. Tracings from projections of the tubules were then made from representative portions of the testes. For this purpose the Mallory triple staining was somewhat more satisfactory. In most cases tracings were made of portions of both of the pair of testes and on a uniform grade of paper; the tubular and intertubular portions were then carefully cut out and weighed. From these weights the percentage of each type of tissue was determined and approximate actual weights of tubular and intertubular tissues calculated.

RESULTS

Pituitary gonadotrophins. In control birds aged 1.5 mo. (i.e., the age when dosage of other birds was started) an average of 57% of intertubular tissue was found in 8 testes; in other control birds killed 16 days later (i.e., when 2.0 mo. old, and at the same time that birds given longest dosage were killed) an average of 55% intertubular tissue was found in 10 testes. The average calculated weights of tubular and of intertubular tissue and average total testes weights obtained on all testes treated for various periods are shown in graphic form in figure 1. The data of the graph indicate that throughout a 16-day period of injection the testes increased in weight at a fairly uniform rate. It is of special interest to observe, however, that the tubular and intertubular elements have an unequal and changing share in this development. During the first 4 days of injection the intertubular tissues developed more rapidly than the ger-

minal elements and at 4 days constituted 63% to 73% of the entire testis. After 4 to 8 days the growth of intertubular elements diminished and indeed it apparently ceased altogether; other available data indicate, however, that its total quantity is slightly greater in these treated testes than is found in normal adult testes. Tubular growth also was accelerated during this same period of continued stimulation, and after 16 daily injections this tissue averaged 73% in 8 heavier testes and 51% in 6 smaller testes. In the group of birds subjected to this longest period of injection an extreme variation was found in size of testis; for the 7 pairs this varied from 296 mg.

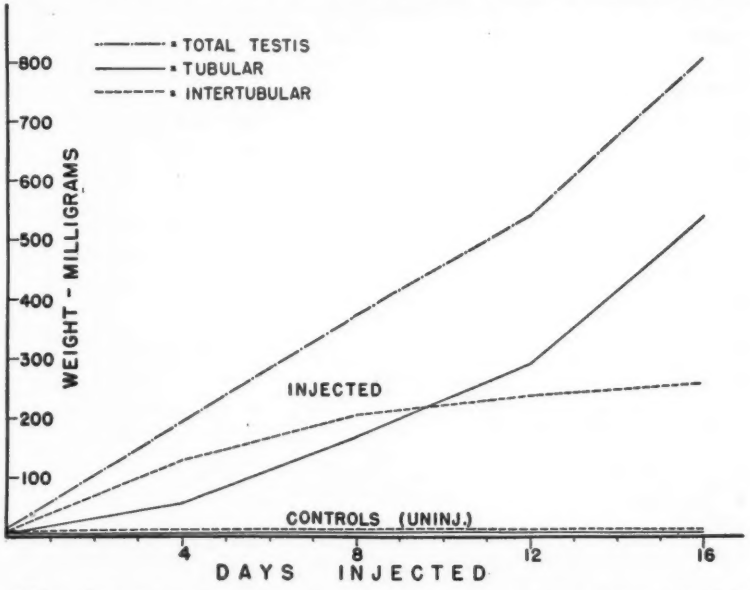


Fig. 1. Average weights of whole testes (pairs), and of their tubular and intertubular elements, based on groups of birds killed after 4, 8, 12 or 16 daily injections with 10 mg. FSH+ 730. Averages for tubular and intertubular portions of the testes of control birds killed at the beginning and end of the period of dosage are shown at the bottom of the figure.

to 1275 mg. Uninjected control testes examined at the end of this 16-day period were almost quiescent and still had an average of 55% of intertubular tissue.

The weights of testes obtained after each period of dosage showed much variation, as did percentage of tubular and intertubular tissue, and a cursory examination suggested that proportions of tubular and non-tubular elements might conform more closely with total weight of testis than with number of days injected. Relative amounts of tubular and intertubular tissue were therefore calculated for all individual pairs of testes and these results, together with a clearer picture of the above-noted variations, are shown in figure 2. The general trend of development of the two types of tissue is indicated by the two lines freely drawn on the graph. The tendency of the intertubular tissues to develop more rapidly during the first days of stimulation and to lag in a later period is again quite evident. It is also notable that a slower development of the tubules permits this tissue to attain approximately 50% of the total in a pair of testes whose total weight is about 500 mg.; thereafter tubular tissue becomes a still higher proportion of the gland.

A similar study was made of the normal growth of tubular and intertubular

portions of the testes of 33 control uninjected pigeons whose gonad weights covered the same range as those present in the injected birds at the beginning and end of the tests. The results are shown in figure 3. The amount of development which occurred in 16 days under injections (fig. 2) required 87 days when stimulated by the bird's own pituitary. In these glands from untreated birds it can be seen that an early and disproportionate increase in intertubular tissues, followed by a period in which these elements grow very slowly, is the normal condition. Also, when growth of the testis is greatly accelerated by gonadotrophins (as in fig. 2) the early response of inter-

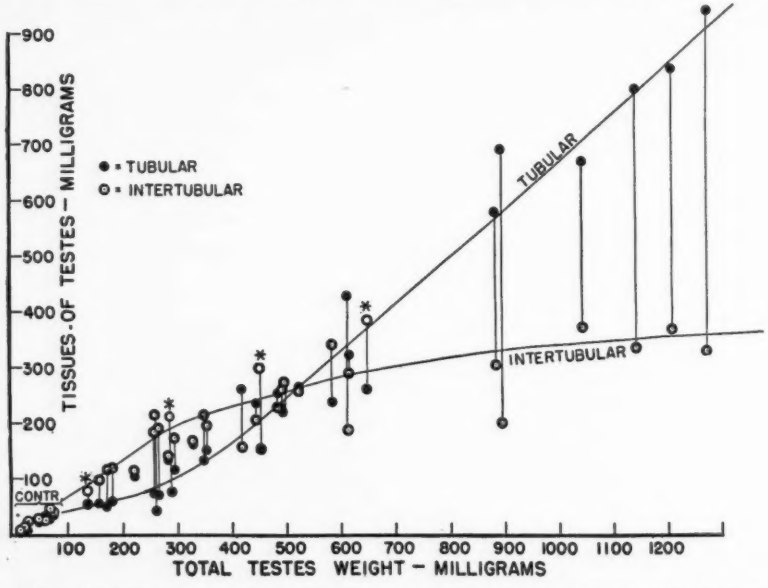


Fig. 2. Relative weights of tubular and intertubular tissues of individual pairs of testes plotted against the weight of the testes of birds injected with preparation (FSH+) 730. The 4 starred values were obtained with LH.

tubular tissues is not only exaggerated but it is maintained at a relatively high level throughout the course of dosage. Tubular development is somewhat slower in both the younger controls (smaller testes) and during the first 4 or 5 days of injection with gonadotrophin. Later, however, the tubular element in both normal and injected groups increases disproportionately and finally constitutes almost the whole of later growth of the mature testis. Involuted mature testes of wild birds were found by van Oordt and Bruyns (19) to show relatively greater atrophy of tubules.

The results obtained from four significant tests made with luteinizing hormone (pituitary LH) kindly supplied by Dr. H. L. Fevold are also included in figure 2. Tested in moderate or low dosage (1.0 or 3.0 mg./day) this LH stimulated growth in both portions of the testis, but in all cases stimulation was greatest in the intertubular elements. Two pairs of testes which weighed less than 300 mg. fit well into the curves obtained for stimulation with our FSH+ preparation 730; in heavier testes, however, a greater amount of intertubular growth and somewhat less of tubular development was obtained after LH than was found in testes of the same weight after FSH+ 730.

Histology of pigeon testes. Histological examination of testes stimulated during

only 4 or 5 days by a wide range of gonadotrophic preparations indicate that the enlargement is due almost entirely to cell hypertrophy and perhaps to increased vascularity (fig. 4, 5, 6). Since the intertubular elements develop most rapidly during this period the hypertrophy is there more pronounced, but an increase in size of the spermatogonial cells indicates that the early growth of tubular tissue likewise consists in an increase in cell cytoplasm (fig. 6, 7, 8). Employment of the colchicine technic in many of the young Carneau pigeons used in this study has failed to show any hyperplasia of mature interstitial cells at any stage of stimulation. If, as seems prob-

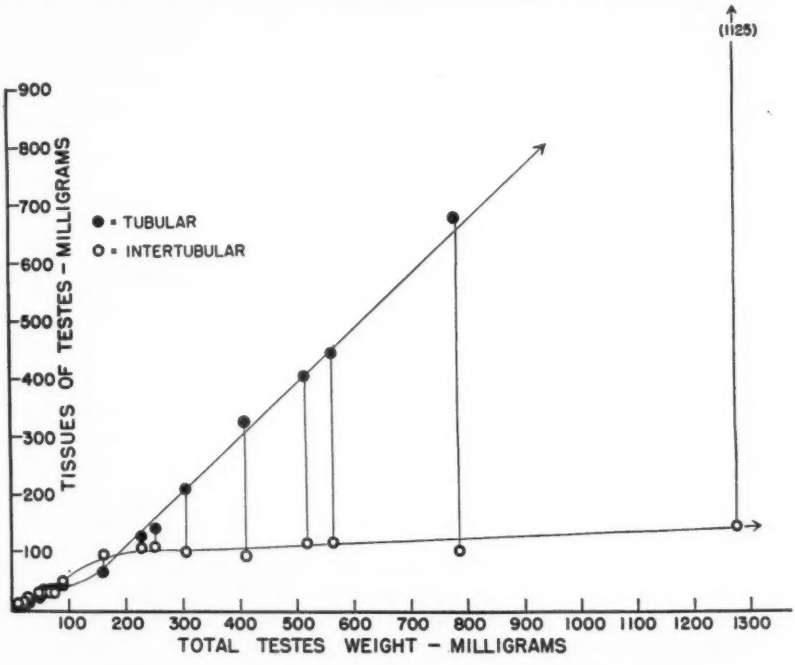


Fig. 3. THE NORMAL DEVELOPMENT OF TESTICULAR TISSUES IN UNINJECTED PIGEONS. Weights of testes of these controls correspond to the range of weights found in the test birds (fig. 2) at the beginning and end of their treatment.

able, interstitial cells originate through differentiation of mesenchymal cells of the tubule wall the absence of mitoses in the fully formed tissue is not remarkable since the observed increase in tissue could thus still arise in part from an increase in cell number (additions of mesenchymal cells in which mitosis is observed with difficulty) and from cell hypertrophy which clearly occurs.

Between the 4th and 8th days of stimulation numerous mitoses appear in the germinal tissue and between the 12th and 16th days spermatids and a few spermatozoa can be found (fig. 10). In the fully mature testis, which is two to three times heavier than that shown in figure 10, the intertubular tissue is relatively greatly reduced but its total quantity is found by calculation to be slightly in excess of that found at earlier stages of maturity.

Histology of testes of Ring doves. The chief histological difference between testes of immature doves and pigeons consists in the presence of a much more compact ar-

rangement of tubules in dove testes due to a minimum of intertubular tissue (fig. 11). In the dove this minimum of intertubular tissue seems to be composed mainly of connective tissue cells. Potent gonadotrophic preparations will increase the weight of dove testes up to 10 to 15 times their control value during a 4-day test. The change in weight is due partly to an increase of 2 to 4 times in the areas occupied by the tubules but more significantly to the formation of variable but large fluid filled intertubular areas (fig. 12). After 4 daily injections the increase of tubule area is ascribable less to increase in cell number than to increased size of spermatogonial cells. Using comparable dosage we have obtained fairly similar or identical histological pictures in dove testes stimulated by (*a*) our numerous pituitary FSH+ preparations, (*b*) by the FSH or LH preparations of Fevold and Hisaw (*c*) by pregnant mare serum, (*d*) by whole pituitary extracts, (*e*) by the urine of a patiant with teratoma testis (see Evans, Simpson. Austin and Ferguson; 20), and (*f*) by better preparations of pregnancy urine (see below).

In doves, as in pigeons, numerous mitoses occur in tubules of birds treated for periods longer than 4 days. Measurements of tubules after 7 days of treatment (fig. 17) indicate an increase to about ten times the area of control tubules; this is associated with a notable increase in number of germinal cells and a contraction of the fluid-filled interspaces. After injection of either an FSH or an LH preparation for 11 days the tubules are well developed and spermatogenesis has advanced to the formation of spermatids (fig. 15). Such testes assumed the appearance of the mature gonad, and there is no evidence of interstitial cell masses such as are seen at this stage in the stimulated testis of the pigeon. The intertubular spaces which were so prominent after stimulation for 4 days nearly disappeared after 13 days injection with pregnant mare serum (fig. 14). In this case testis weight was increased by 28 to 30 times. A single tubule here had about 16 times the area of a control tubule and is approximately of adult size. Many spermatids and some spermatozoa were present (fig. 13, 14).

Extra-pituitary gonadotrophins. Riddle and Polhemus (3) reported the failure of the pregnancy urine (PU) preparations available in 1929–31 to stimulate the testes of doves and pigeons and concluded that its action is distinct from that of the pituitary principle. Similar failures were reported in fowl (21, 22), fowl and duck (5), pigeons (7) and English sparrows (23). Since all the preparations used in these apparent failures successfully stimulated mammalian ovaries it has usually been considered that testes of birds clearly differentiate between gonadotrophins of pituitary and urinary origin. This situation is markedly changed and partially clarified by the data presented herewith.

Fig. 4. Normal testis of pigeon aged 1.5 mo. Weight (2 testes), 22.3 mg. × 130.

Fig. 5. Normal testis of pigeon aged 2.4 mo. Weight (pair) 77.9 mg. × 130.

Fig. 6. Testis of pigeon aged 2.2 mo. after 4 days injection with 3 mg. per day of LH (no. 28C11). These testes weighed 650 mg. and were 59.3% intertubular tissue. Note hypertrophy of interstitial cells. × 130.

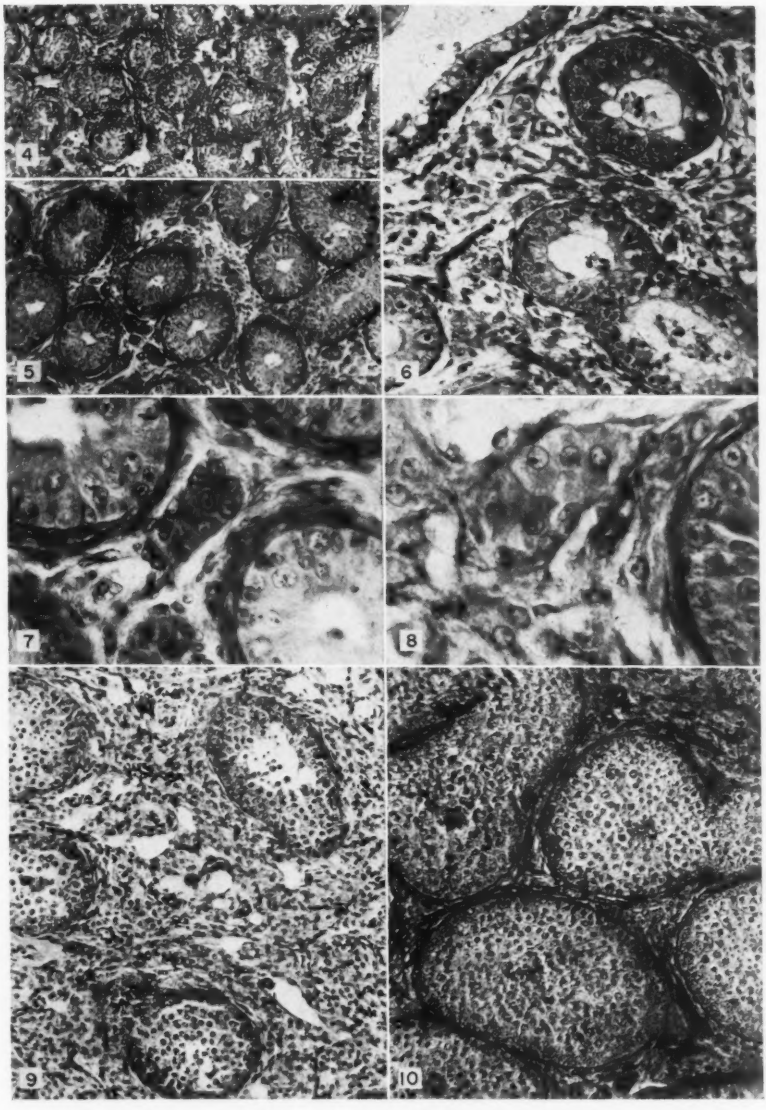
Fig. 7. Higher magnification of same testis as in figure 5. Showing normal condition of connective tissues, a cluster of Leydig cells, and the immature state of the germinal cells. × 420.

Fig. 8. Higher magnification of the LH stimulated testis shown in figure 6. Note hypertrophy of all cells, and especially of a cluster of Leydig cells. × 420.

Fig. 9. Testis of pigeon showing excessive interstitial stimulation produced by 15 daily injections, 1.0 mg. per day, of LH (no. 28C11), and also marked stimulation of the germinal tissues. The two testes weighed 455 mg. and were 66% intertubular tissue. × 130.

Fig. 10. Testis of pigeon showing results of 16 daily injections, 10 mg. per day, of FSH+ 730. The two testes weighed 1.40 gm. and were 70% tubular tissue. Note the advanced stages of spermatogenesis; a few spermatozoa were found in some tubules. × 130.

(All testes figured are from young Carneau pigeons)



Figs. 4–10. *See opposite page for legend.*

Certain PU preparations made during and after 1933 have been sufficiently concentrated and purified to permit clear demonstrations of their ability to stimulate the testes of immature doves and pigeons. Typical results from the numerous tests made by us (1933 to 1940) with these more recent preparations, and hitherto only briefly reported (24), are brought together in table 1. Some of these newer preparations were tested and found to be effective in hypophysectomized pigeons. In tabulating

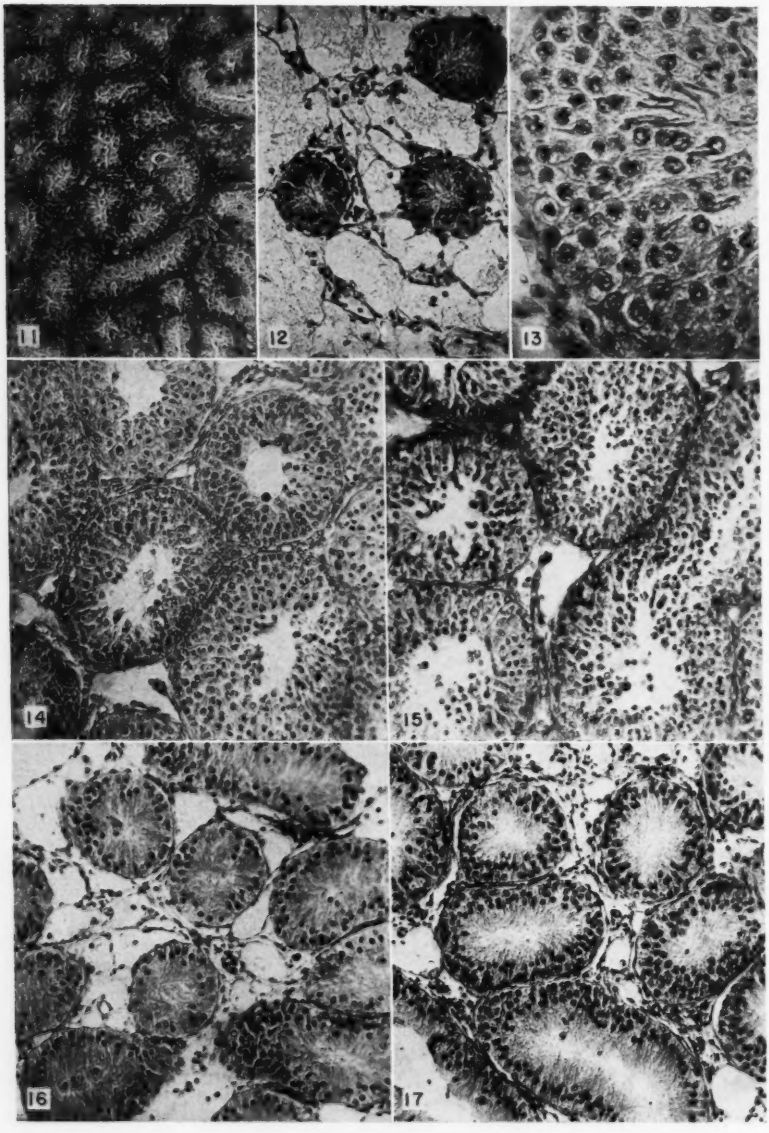


Fig. 11. Normal testis of ring dove aged 2.3 months. The two testes weighed 6.9 mg.; there is little or no intertubular tissue. ×130.

Fig. 12. Testis of dove after 4 daily injections (0.2 cc./day) with serum of pregnant mare. The two testes weighed 69.7 mg. The excessive formation of intertubular fluid-filled areas is shown. The amount of intertubular tissue is small. ×130.

Fig. 13. Part of a tubule from testis of dove injected for 13 days with serum of pregnant mare (0.2 cc./day). Note spermatogenesis and the presence of spermatozoa. ×355.

Fig. 14. Testis of dove, same as figure 13. Intertubular fluid-filled spaces have practically disap-

'control' data for testes of hypophysectomized birds two values are given; the value first given (in parentheses) represents weight of testes at the time hypophysectomy was performed (or when dosage was begun) while expected weight of testes at autopsy in untreated hypophysectomized animals is given second. It is notable that longer periods (7 to 10 days) of dosage are far more effective than 4-day periods, and also that larger doses produce greater effects. Again, a low but measurable response of the dove testis to PU is shown by various calculations involving minimal ovulation doses (M.O.D.) of the same preparations in New Zealand rabbits aged 4 months. For example, 175 R.U. (25 R.U. daily for 7 days) of antuitrin-S increased testis weight in 4 doves by 245%, an amount which corresponds to 4.2% increase in weight of testis for each M.O.D. administered.

In general, the histology of dove testes stimulated with prolan or antuitrin-S does not differ markedly from that found after pituitary gonadotrophins, LH particularly. Following injection in Ring doves for 4 days the intertubular spaces are highly developed and a moderate increase of tubular areas is found. After 10 days of treatment intertubular spaces may have disappeared coincident with a great expansion of tubular tissue, or there may be testicular injury accompanying a marked increase in testis weight. In the latter case intertubular spaces may largely persist and the much expanded tubular walls sometimes become thin and definitely abnormal. In terms of milligrams most of these PU preparations still require higher dosage than with pituitary gonadotrophin and they still appear to contain substances which are definitely toxic to the bird. In hypophysectomized pigeons PU has not been observed to stimulate testes greatly, but considerably more than testis maintenance resulted from low or moderate dosage.

The placental (APL) extracts tested, like some untabulated 4-day tests with menopausal urine (confirming Evans and Simpson, 7), proved ineffective in the dosage used; it is quite possible, however, that these failures (table 1) have the same questionable basis as the many earlier failures with PU. Certainly gamone (from menopausal urine) fully sustained testis weight in hypophysectomized young pigeons though the toxicity of the preparation permitted only relatively low dosage.

Hamburger (25) showed that the serum of pregnant mares effectively stimulates the testes of young cocks. Evans and Simpson (7) injected an extract of the mare's endometrium into young pigeons (of 4 or 5? wk.) and concluded that "the testes of such birds is not a sensitive test object for the gonadotrophic hormone present in the blood stream of mares in early pregnancy." When body weight is taken into consideration it does not appear, however, that the results obtained by Evans and Simpson with an extract of endometrium indicate less sensitivity in pigeon testis than in rat ovary. Data supplied in table 1 show that in one type of dosage (0.4 cc. daily for 7 days) each rabbit M.O.D. (0.15 cc.) of true mare serum produced nearly

peared, few Leydig cells are present, and sperm production has been started in a pair of testes whose combined weight was 19.4 mg. ×130.

Fig. 15. TESTIS OF A DOVE AFTER 11 DAILY INJECTIONS (4.0 MG./DAY) OF LH $11R_3$, FROM FEVOLD AND HISAW. The testes weighed 116.8 mg. Gametogenic potency is shown by the very advanced development of the germinal tissue. Fluid-filled intertubular spaces have almost disappeared and there is very little intertubular tissue. ×130.

Fig. 16. TESTIS OF A DOVE AFTER 4 DAILY INJECTIONS (2 R.U./DAY) WITH A PURIFIED (FEVOLD AND HISAW) PITUITARY FSH PREPARATION. The two testes weighed 72.6 mg. Note the fluid-filled interstices and hypertrophy of spermatogonial cells. ×130.

Fig. 17. TESTIS OF A DOVE AFTER 7 DAYS OF THE SAME DOSAGE USED IN FIGURE 16. Weight of two testes, 132.8 mg. Note the diminution of fluid-filled areas, further hyperplasia of germinal cells, and the pronounced enlargement of tubules in comparison with controls (fig. 11). Many tubule cells are in process of division. ×130.

(All testes figured are from young Ring doves aged 1.5 to 2.5 months)

TABLE 1. MORE RECENT TESTS OF EXTRA-PITUITARY (AND OF PITUITARY) GONADOTROPHINS ON TESTES OF IMMATURE DOVES AND PIGEONS

Birds used		Dosage		Average age (from hatching)	Weight of testes		M.O.D. in rabbit
Description	No.	Daily amount	No. of days		Tests	Control	
Antuitrin-S (Parke, Davis & Co.)							
Ring doves	2	10 R.U.	4	1.9	8.2	7.0	3.0 R.U.
Ring doves	2	30 R.U.	4	1.6	8.6	6.1	3.0 R.U.
Ring doves	4	20–30 R.U.	7	2.3	29.3	8.6	3.0 R.U.
Ring doves	2	50–100 R.U.	4	1.9	22.7	7.0	
Ring doves	5	50–80 R.U.	7	2.2	82.6	8.2	
Ring doves	2	100 R.U.[1]	8	2.6	167.1[1]	9.8	
Follutein (Squibb & Sons)							
Ring doves	2	50 R.U.	7	2.2	22.8	8.2	
Prolan (I.G., Elberfeld, Germany)							
Ring doves	4	15–60 R.U.	4	1.9	6.5	7.0	7.5 R.U.
Ring doves	2	75 R.U.	7	2.0	17.4	5.7	7.5 R.U.
Ring doves	1	750 R.U.	4	1.9	30.1	7.4	7.5 R.U.
Ring doves	2	750 R.U.	10	1.9	60.1	7.4	
Ring doves	2	5500 R.U.	4	2.2	65.2	8.2	
Hypct. Car. pigeons	9	300 R.U.	10	1.8	88.9	(33) 22	
Hypct Car. pigeons	1	1050 R.U.	10	2.4	153.2	(22) 22	
Pregnyl (Organon, Holland)							
Ring doves	2	1.2 mg.	4	2.1	57.5	7.8	
Gamone (Squibb & Sons)							
Hypct. Car. pigeons	6	3.0 mg.	10	1.8	61.3	(33) 22	
Hypct. Car. pigeons	4	1.5 mg.	10	1.8	54.4	(33) 22	
A.P.L. from placenta (Ayerst, McKenna & Harrison; and own, last)							
Ring doves	1	8 R.U.	4	1.7	3.4	6.4	9.0 R.U.
Ring doves	4	10 mg.	7	2.0	6.8	7.4	7.5 mg.
Pregnant Mare Serum							
Ring doves	2	0.6 cc.[1]	4	2.1	30.5[1]	6.0	0.15 cc.
Ring doves	2	0.4 cc.	7	1.9	135.1	7.0	0.15 cc.
Ring doves	1	0.2 cc.	13	1.9	194.3	7.0	0.15 cc.
Mare Serum Powder							
Ring doves	2	4 R.U. (10 mg.)	4	1.8	30.1	6.7	
Gonadogen (Upjohn Co.)							
Hypct. Car. pigeons	6	2 R.U.	10	1.9	201.1	(38) 22	
Hypct. Car. pigeons	6	2 R.U.	10	1.8	175.2	(36) 22	
Pituitary LH (Fevold and Hisaw; Wallen-Lawrence, 5 mg. test)							
Ring doves	3	2 mg.[1]	4	2.0	44.0[1]	7.4	0.35 mg.
Ring doves	2	3 mg.	4	1.9	78.1	7.0	(?)
Ring doves	1	4 mg.	11	1.9	116.8	7.0	0.35 mg.
Ring doves	3	5 mg.	4	2.0	83.2	7.4	
Carneau pigeons	3	3 mg.	4	2.2	357.9	75.0	
Carneau pigeons	1	1 mg.[1]	15	2.0	455.0[1]	63.0	
Pituitary FSH (Fevold and Hisaw)							
Ring doves	4	2 R.U.	4	2.0	60.9	7.4	
Ring doves	1	2 R.U.(?)	7	2.3	132.8	8.2	
Own Pituitary FSH+							
Ring doves	4	1 mg.	4	1.9	30.0	7.0	0.17 mg.
Ring doves	8	2 mg.	6	1.9	89.2	7.0	
Hypct. Car. pigeons	6	2 mg.	10	2.1	341.0	(50) 22	
Hypct. Car. pigeons	3	3.1 mg.	10	1.8	443.0	(33) 22	
Own Whole Anterior Pituitary Extract							
Hypct. Car. pigeons	2	10 mg.	10	1.8	520.0	(33) 22	

[1] Injected twice daily.

100% increase in testis weight in Ring doves. These data demonstrate very great sensitivity of the dove testis to mare serum, though it does not follow necessarily that doves and pigeons are more suitable than other animals for the assay of this hormone.

Data concerning the testis stimulating ability of special types of pituitary gonadotrophins are also given in table 1. It is clear that purified FSH and LH both rapidly increase the weight of testes of doves and pigeons, and the data given here supplement the information given in figures 6, 8, 9, 15, 16 and 17 concerning the action and effectiveness of these hormones. Additional data in the table indicate the relatively high potency in doves and hypophysectomized pigeons of three of our own preparations (similar to 730) which we call FSH+.

DISCUSSION

Breneman (13) has earlier noted that both testes and ovaries of chicks aged 5 to 15 days are highly sensitive to mare serum. It is also notable that in this same study Breneman expressed doubt that the PU preparations used by him had failed to produce some stimulation in the testes of immature chicks. Pituitary LH fractions similar to those used by us (table 1) increased the weight of immature chick testes only moderately in comparison with FSH and, according to Breneman (14), this stimulation was confined largely or solely to intertubular elements. Asmundson and Wolfe (26) observed that mare serum stimulates the testes of immature chicks and turkeys. Cole (27) has especially stressed the fact that the nature of the response of the immature rat ovary to a single injection of mare serum depends upon the age of the rat and the size of the dose.

Six of the PU preparations submitted early in 1938 to the Third International Conference on Standardization of Hormones were assayed by means of their degree of stimulation of the dove testis and the results published (28) along with assays made by other methods in other laboratories. The dove testis test showed that one of these six PU preparations, though made as late as 1937 or 1938, was extremely weak in gonadotrophin; practically all other tests by other methods likewise indicated its exceptionally low potency and it (and it only) was excluded from the pool of preparations which constitute the International Standard for chorionic gonadotrophin. Again, the relative order of potency of those six PU preparations (as this was determined by the combined results from several methods of assay in various laboratories) was fairly closely approximated by the tests on doves although only a single immature male dove was used for the assay of each PU preparation.

The data of table 1 clearly show that two errors have attended the earlier reports of failure of PU to stimulate bird testes. First, it was wrongly assumed that adequate amounts of hormone had been used but with no response. Second, this failure has been widely regarded as providing evidence or proof for a qualitative difference between urinary and pituitary gonadotrophin. On other grounds one may admit or deny that the two types of preparations are qualitatively different; but the point emphasized here is that it is no longer possible to use unresponsiveness of the bird testis as an evidence for a qualitative difference. Current PU preparations usually still contain material that is toxic but they exert a well marked and fairly characteristic gonadotrophic action on the testes of immature doves and pigeons.

During the first 4 days of injection of FSH+ into immature male Carneau pigeons the high pitched voice of the young bird changes to the coarser growl of the adult. This suggests that the hypertrophy which occurs in interstitial cells prior to 4 days in these birds coincides with the period at which these cells begin the production of appreciable amounts of androgen and the masculinization of the young bird.

The first response of the testes of pigeons to gonadotrophins is an hypertrophy of both interstitial and spermatogonial cells while a hyperplasia of germinal tissue begins after 4 to 6 days; this general type of response occurs under the stimulus of either FSH or LH, and in large measure it represents the order of occurrence of changes in the normal growth of the pigeon testis.

Since intact pigeons were used in the tests made with preparation 730 the results of those tests permit question of the extent to which the bird's own pituitary entered into the development of the tubular or intertubular testis. There is further possibility that the intact pituitaries were modified by the course of injections. Riddle and Schooley (29) supplied evidence that pituitaries of similar immature pigeons have little or no gonadotrophic potency (as this is measured by the response of the immature dove testis to implants of the pituitaries of immature doves and pigeons). It has also been shown (30) that the decreased weight of testes of immature pigeons following hypophysectomy is at least largely the result of reduced food intake since testes of unoperated pigeons diminish in size when the food supply is limited. There is further possibility that apparently inactive pituitaries might produce sufficient gonadotrophic substance to synergize injected material and thus play a part in the results obtained in this study. On this point it may be noted that Evans and Simpson (7) failed to get evidence of synergism of pituitary gonadotrophin by PU in pigeons, and Breneman (13) found no synergism of FSH by LH in chicks.

Well marked structural differences exist between the intertubular tissues of pigeons and those of doves. At no time are Leydig cells prominent in the dove's testis. The fluid which fills the intertubular spaces of the stimulated testis of the dove has the appearance of lymph or plasma; its presence may be the result of the hyperemia which accompanies rapid stimulation and which in extreme cases results in hemorrhage.

SUMMARY

Daily injections of a gonadotrophic pituitary extract practically free of prolactin into 32 Carneau pigeons aged 1.5 months during periods of 4, 8, 12 and 16 days resulted in rapid and progressive growth and maturation of their testes. For all testes obtained at the above-mentioned periods of treatment the percentage of tubular and intertubular tissue was determined and from these values the total weight of each type of tissue was calculated.

Histological study showed that during the first 4 to 6 days of treatment a marked hypertrophy of intertubular, interstitial and germinal tissues occurred but with a lesser relative increase in the tubular tissue. Under more prolonged dosage growth is almost wholly restricted to tubular tissue and spermatogenesis is initiated; intertubular tissues, however, tend during continuance of dosage to remain at a relatively higher level than in testes which mature normally without injections.

In 33 control Carneau pigeons whose testis weight covered the same range as those of all injected birds, and whose ages ranged from 1.4 to 4.3 months, the phase of overgrowth of tubules was found to occur after the intertubular tissues had made most of their growth. Thus testis development under injections of gonadotrophin (FSH particularly) roughly parallels the normal development.

In immature testes of pigeons and doves the interstitial tissues make greater response during the first 4 days to purified LH than to FSH but after 7 to 14 days of treatment all tissues are rather similarly developed by FSH and LH.

Testes of pigeons contain a greater proportion of interstitial (Leydig) cells than do testes of Ring doves. Testes of doves differ from those of pigeons by forming large fluid filled spaces in all intertubular areas in response to high dosage with gon-

adotrophin for 3 to 5 days. During more prolonged dosage these spaces diminish and by the time mature sperm are formed they completely disappear.

The testes of immature doves and pigeons are highly sensitive to the gonadotrophins contained in serum of pregnant mares.

Testes of immature Ring doves show rather similar gametogenic stimulation after injection with such dissimilar preparations as pituitary FSH, LH, pregnant mare serum, human pregnancy urine, and urine from a patient with teratoma testis.

Gonadotrophins from pituitary, mare serum and human pregnancy urine, all stimulate growth in testes of hypophysectomized pigeons. Contrary to earlier evidence and current belief the immature testes of doves and of normal and hypophysectomized pigeons respond by marked growth and maturation to newer, purer, and less toxic preparations of human pregnancy urine. Conclusions based upon an alleged inability of the bird testis to respond to PU preparations must be reconsidered.

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REFERENCES

1. Riddle, O., and F. Flemion: *Am. J. Physiol.* 87: 110. 1928.
2. Riddle, O.: *Endocrinology* 25: 307. 1931.
3. Riddle, O., and I. Polhemus: *Am. J. Physiol.* 98: 121. 1931.
4. Schockaert, J. A.: *Anat. Rec.* 50: 381. 1931.
5. Schockaert, J. A.: *Am. J. Physiol.* 105: 497. 1933.
6. Domm, L. V., and H. B. van Dyke: *Proc. Soc. Exper. Biol. & Med.* 30: 349. 1932.
7. Evans, H. M., and M. E. Simpson: *Anat. Rec.* 60: 405. 1934.
8. Fevold, H. L., F. L. Hisaw and S. L. Leonard: *Am. J. Physiol.* 97: 291. 1931.
9. Wallen-Lawrence, Z.: *J. Pharmacol. & Exper. Therap.* 51: 263. 1934.
10. Greep, R. O., H. L. Fevold and F. L. Hisaw: *Anat. Rec.* 65: 261. 1936.
11. Greep, R. O.: *Cold Spring Harbor Symp. Quant. Biol.* 5: 136. 1937.
12. Greep, R. O., and H. L. Fevold: *Endocrinology* 21: 611. 1937.
13. Breneman, W. R.: *Anat. Rec.* 64: 211. 1936.
14. Breneman, W. R.: Indiana Univ. Publication, Science Ser., No. 7. 1938.
15. Lahr, E. L., O. Riddle and R. W. Bates: (Abst.) *Am. J. Physiol.* 116: 94. 1936.
16. Bates, R. W., and O. Riddle: *J. Biol. Chem.* 123: v. 1938.
17. Bates, R. W., O. Riddle and R. A. Miller: *Endocrinology* 27. 781. 1940.
18. Evans, H. M., H. Fraenkel-Conrat, M. E. Simpson and C. H. Li: *Science* 89: 249. 1939.
19. van Oordt, G. J., and M. F. M. Bruyns: *Ztschr. Morphol. u. Ökol. der Tiere* 34: 161. 1938.
20. Evans, H. M., M. E. Simpson, P. R. Austin and R. S. Ferguson: *Proc. Soc. Exper. Biol. & Med.* 31: 21. 1933.
21. Reiss, M., R. Pick and K. A. Winter. *Endokrinol.* 12: 18. 1933.
22. Martins, T.: *Mem. do Inst. Butantan* 8: 349. 1934.
23. Witschi, E., and W. N. Keck: *Proc. Soc. Exper. Biol. & Med.* 32: 598. 1935.
24. Riddle, O., and E. L. Lahr: (Abst.) *Anat. Rec.* 78: 88. 1940.
25. Hamburger, C.: *Endokrinol.* 13: 305. 1934.
26. Asmundson, V. S., and M. J. Wolfe: *Proc. Soc. Exper. Biol. & Med.* 32: 1107. 1935.
27. Cole, H. H.: *Am. J. Anat.* 59: 299. 1936.
28. Third International Conference on Standardization of Hormones. Bull. Health Organ., League of Nations 8: 868. 1939.
29. Riddle, O., and J. P. Schooley: *Proc. Soc. Exper. Biol. & Med.* 32: 1610. 1935.
30. Schooley, J. P., O. Riddle and R. W. Bates: *Am. J. Anat.* (In press).

EFFECTIVENESS OF HEME IN THE AUGMENTATION OF GONADOTROPIC EXTRACTS FROM DIFFERENT SOURCES[1]

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SEVERAL REPORTS have been made which show the increased effectiveness of gonadotropic hormones of the anterior pituitary gland when injected with other substances which are inactive as regards the stimulation of the ovary of the immature rat. These augmenters include tannic acid (1), copper (2), and zinc salts (3, 4, 5), yeast extract and ash from yeast (2), male urine (6), blood serum (7), leucocytes (8), egg albumin (9), merthiolate (10) and chlorophyll (11, 12). Casida (13) reported that the formed elements of blood augment gonadotropic extracts of sheep pituitary powder and that the ash from the formed elements does not. McShan and Meyer (14) separated the hemoglobin from the blood of the cow into heme and globin, and found that the hemin was an effective augmenter when it was dissolved in dilute sodium carbonate or sodium hydroxide solution and mixed in vitro with gonadotropic extract of sheep pituitary powder. Later Breneman (12) reported that extracts of hog pituitary glands were augmented by heme when sufficient pyridine was added to the mixture to dissolve the hemin, and Evans, Hines, Ceithaml and Koch (15) reported that extracts of sheep pituitary glands were augmented by hemin.

Since heme was found to be an effective augmenter of gonadotropic extracts of sheep pituitary powder, it was of interest to test the effectiveness of heme in the augmentation of gonadotropic extracts prepared from the pituitary glands of other animals, from urine of pregnant women, and from the serum of pregnant mares. Various other substances were tested for their ability to augment gonadotropic hormone extracts. It was also of interest to study the heme-gonadotropic complex from the standpoint of the stability of the union between the heme and the gonadotropic hormone.

MATERIALS AND METHODS

The source of the gonad stimulating materials used in this study were: human, horse, hog, sheep and cow pituitary glands which were dehydrated with acetone; prephysin, a commercial pituitary gonadotropic extract; and follicle stimulating preparations (FSH) made from sheep pituitary powder by a modification of the Fevold method (16), and by the trypsin method (17); two extracts prepared from the urine of pregnant women; and the serum of pregnant mares in the untreated and purified forms (18). The human glands were given in the form of desiccated powder suspended in water while the glands of the horse were given in the form of the acetone dried powder suspended in water, and as an aqueous extract of the dried powder. Aqueous extracts of the acetone dried pituitary powder of the cow and the hog were used. One of the preparations of the urine of pregnant women was made by precipitation with alcohol while the other was purified by precipitation with brucine (19).

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The gonadotropic hormone preparations were assayed by use of immature female rats which were 21 days old on the day the injections were begun. The same quantities of the extracts were tested after mixing in vitro with the heme obtained by dissolving 4 mg. of hemin in dilute sodium hydroxide or dilute sodium carbonate solution. The hemin was prepared from the blood of the cow by the method of Drabkin and Austin (20). Hemin prepared from the blood of the horse was effective also. The gonadotropic extracts and the mixture of heme and gonadotropic extracts were assayed by subcutaneous injection into 21-day-old rats of 0.5 cc. of the proper extract on the afternoon of the first day and twice daily with this amount on each of the 4 succeeding days. The animals were killed on the morning of the sixth day and the ovaries were removed, weighed and examined in the fresh condition for the presence and relative numbers of follicles and corpora lutea.

The stability of the union between the heme and the gonadotropic hormone of sheep pituitary extract was studied by precipitation of the complex with acid followed by washing with water, and by electrodialysis. The hemin was dissolved in dilute sodium hydroxide solution and thoroughly mixed with the gonadotropic extract by shaking after which it was cooled for one hour. The solution of the heme-gonadotropic complex was adjusted to pH 5 by the addition of dilute hydrochloric acid. It was found also that this complex precipitated at a pH as great as 6.5. The precipitate of the complex which was obtained at pH 5 was recovered by centrifugation and suspended in a definite volume of water for testing. Precipitates of heme and purified pregnant mare serum, and of heme and extract of pregnant urine were prepared similarly.

The precipitate of the heme-gonadotropic sheep pituitary complex was washed repeatedly for as many as 5 times. During the washing sufficient acid was added to keep the pH below 6.0. The washed precipitates were tested to determine whether the active substance remained associated with the insoluble heme.

The solution of the heme-gonadotropic complex was electrodialyzed. Membranes made of cellophane were used, and after the electrodialysis had been continued for approximately 5 hours a precipitate formed. The precipitate was recovered by centrifugation after which the supernatant liquid was clear. Both the precipitate and the supernatant liquid were tested for gonadotropic activity.

Other compounds and substances were tested for their ability to augment the gonadotropic hormone contained in sheep pituitary extracts. These substances were dissolved in the extract and assayed by the standard procedure.

RESULTS AND DISCUSSION

The data in table 1 give the average weights of the ovaries obtained by administration of the gonadotropic extracts alone as compared to those obtained by the administration of the extracts with heme. The gonadotropic extract of human pituitary glands was augmented by heme when it was administered in the form of an aqueous suspension of the acetone powder. The aqueous extract of hog pituitary powder was augmented by heme, but not to the extent as were similar extracts of sheep pituitary powder (14). Evans, Hines, Ceithaml and Koch (15) reported also that sheep pituitary extract was augmented by hemin while Breneman (12) reported that hog pituitary extract was augmented by hemin when in solution in pyridine. The augmentation reported by these workers was small, however, as compared to that obtained in this laboratory.

Ovaries produced by the mixture of a solution of heme with an aqueous suspension of acetone dried powder of horse pituitary glands had an average weight of 52 mg. as compared to 36 mg. for the suspension of the acetone powder given without the

heme. This difference in the average weights of the ovaries, however, probably does not indicate that there was augmentation as the weights of the ovaries produced by the heme-pituitary mixture varied from 29 mg. to 113 mg. while there was little variation in the weights of the ovaries when the pituitary powder was given alone. The ovaries produced by an aqueous extract of horse pituitary powder had an average weight of 38 mg. as compared to 20 mg. when the heme-extract solution was given.

TABLE 1. EFFECT OF HEME ON THE ACTIVITY OF GONADOTROPIC HORMONE PREPARATIONS

Source of preparation	Kind of prep.	Dose prep.	Hemin	No. of rats	Av. wt. ov.
			mg.		*mg.*
Pituitary powder					
1. Human	Aqueous suspension	5 mg.	—	6	28
		5 mg.	4	6	126
2. Hog	Aqueous extract	100[1]	—	3	30
		100[1]	4	3	121
3. Horse	Aqueous suspension	10 mg.	—	6	36
		10 mg.	4	12	52
	Aqueous extract	10[1]	—	4	38
		10[1]	4	6	20
4. Cow	Aqueous extract	300[1]	—	6	13
		300[1]	4	6	102
5. —	Prephysin	0.4 cc.	—	4	43
		0.4 cc.	4	3	153
6. Sheep	(1) FSH	300[1]	—	3	28
		300[1]	4	3	97
		500[1]	—	7	41
		500[1]	4	6	148
	(2) FSH (trypsin method)	500[1]	—	9	44
		500[1]	4	9	88
7. Pregnant mare serum	Untreated	0.1 cc.	—	6	29
		0.1 cc.	4	6	23
	Gonadogen	1 U	—	6	22
		1 U	4	9	25
8. Pregnancy urine	Brucine prolan	2.25 mg.	—	6	24
		2.25 mg.	4	6	29
	Alcohol precipitate	23.3 mg.	—	4	40
		23.3 mg.	4	6	41

[1] Milligrams equivalent of pituitary powder.

This shows that augmentation does not result when extracts of horse pituitary glands are given with heme, and Deanesly (4) has reported that zinc sulphate does not augment the gonadotropic hormone of horse pituitary glands.

The heme-hormone precipitate of horse pituitary extract which was obtained by adjusting the solution to PH 5 when given in a dose of 20 mg. equivalent of dried gland produced ovaries which had an average weight of 44 mg. as compared to 16 mg. when 10 mg. equivalent of the precipitate was given. The supernatant liquid from which the heme-hormone precipitate was obtained did not contain activity (table 3, preparation 5). These results show that the hormone of horse pituitary

glands was precipitated from solution with the heme, but that this association of the hormone with the heme did not result in augmentation.

The aqueous extract of the acetone powder of cow pituitary glands when given in a dose of 300 mg. equivalent did not stimulate the ovaries of immature rats while the average ovarian weight of 102 mg. was obtained when the same amount of the extract was given with heme. Prephysin, a commercial pituitary extract, was augmented by heme.

The FSH preparation made from sheep pituitary powder by the method of Fevold (16) was augmented to a greater extent by heme than were ones made by the trypsin method (17). A few of the ovaries obtained by the augmentation of both types of FSH contained corpora lutea. These results indicate that the presence of the luteinizing hormone (LH) is not necessary for the augmentation of FSH by heme as the augmentation is not as great as that produced when unfractionated pituitary extracts which contain large amounts of LH are injected with heme.

TABLE 2. EFFECT OF HEME AND PREGNANCY URINE EXTRACT ON THE GONADOTROPIC ACTIVITY OF SHEEP PITUITARY EXTRACT

Kind of preparation	Pit. ext.	Dose PU	Hemin	No. of rats	Av. ov. wt.
	mg. eq.	*mg.*	*mg.*		*mg.*
SAP72 (Sheep)	25	—	—	4	17
SAP72+heme	25	—	4	7	105
SAP72+PU (P51)	25	2.25	—	3	81
SAP72+PU (P51)	25	4.50	—	3	100
PU (P51)	—	2.25	—	6	24
PU+Heme	—	2.25	4	6	29
SAP72+PU+Heme	25	2.25	4	3	242

The gonadotropic hormone preparation obtained from the serum of pregnant mares (PMS), and the ones prepared from the urine of pregnant women (PU) were not augmented by heme (table 1). The gonadotropic hormones contained in the PMS and PU preparations when dissolved in dilute basic solution with heme were precipitated with the heme when the solution was adjusted to pH 5. There was little gonadotropic activity in the pH 5 supernatant liquid from the PMS while there was none in that obtained from PU. The results from the assays of the precipitates and supernatant liquids are given in table 3 (preparations 6, 7). These results indicate that the gonadotropic hormone of PU, and also of PMS, was precipitated by the heme, but this did not result in the augmentation of these preparations.

The gonadotropic hormone of PU is known to augment gonadotropic extracts of pituitary glands. It was of interest, therefore, to determine whether the effectiveness of gonadotropic extract of sheep pituitary glands could be further increased by administration of a mixture of the extract, heme and PU. The data in table 2 show that the maximum effect of the gonadotropic extract was not obtained when it was given with heme or PU alone as the ovarian weights were greater when the three substances were given together.

It became of interest also to study the stability of the union between the heme and the gonadotropic hormone of sheep pituitary extracts as this complex was highly effective in the stimulation of the ovaries of immature rats. The solution of the heme-gonadotropic hormone complex of sheep pituitary extract was prepared by the usual procedure, and then the heme was precipitated by adjusting the solution to pH 5 with dilute hydrochloric acid. The data given in table 3 (preparation 1) show that the gonadotropic activity was associated with the heme precipitate and that the

supernatant filtrate from which the heme was precipitated contained very little activity. The gonadotropic activity was not precipitated when the extract was adjusted to pH 5 in the absence of heme. It was not possible to separate the hormone from the heme by washing the heme-hormone precipitate as many as 5 times. This treatment did not result in a decrease in the activity of the precipitate (table 3, preparation 2). During the washing the pH of the mixture was not allowed to become greater than 6 so as to prevent solution of the precipitate.

The gonadotropic hormone of sheep pituitary extract was not separated from the heme by precipitation of the complex from the solution with acetone. The hormone was also precipitated with the heme when the solution was electrodialyzed for 5 hours

TABLE 3. THE STUDY OF THE STABILITY OF THE UNION BETWEEN THE HEME AND THE GONADOTROPIC HORMONE

Kind of preparation	Dose		No. of rats	Av. wt. ov.
	Prep.	Hemin		
	mg. eq.	*mg.*		*mg.*
1. Heme-sheep pit. ppt.	50	4	10	243
Heme-sheep pit. ppt.	100	8	3	248
Heme-sheep pit. fil.	50	—	4	19
2. Heme-sheep pit. ppt.				
pH 5. Washed ×1	50	4	3	164
Washed ×2	50	4	7	168
Washed ×3	50	4	6	140
Washed ×5	50	4	9	169
Washed ×1	100	8	7	241
Heme-sheep pit. fil.	50	—	6	19
3. Heme-sheep pit. acetone ppt.	100	8	4	169
4. Heme-sheep pit. ppt. from electrodialysis	50	4	2	173
Electrodialysis fil.	100	—	2	10
5. Heme-horse pit. ppt.	20	4	3	44
Heme-horse pit. fil.	20	—	3	10
6. Heme-PMS ppt. P_3E_1	0.25 mg.	4	3	36
Heme-PMS fil. P_3E_1	0.25 mg.	—	3	22
7. Heme-PU ppt. P_{52}	35 mg.	4	4	31
Heme-PU fil. P_{52}	35 mg.	—	4	12

but it was not separated from the heme by this treatment as this precipitate produced augmentation (table 3, preparations 3, 4).

The fact that it was not possible to separate the gonadotropic activity contained in sheep pituitary extracts from the heme by the above methods indicates that the hormone is firmly held by the heme. The results do not demonstrate whether this union is physical or chemical in nature. However, the augmentation of the gonadotropic hormone by addition of insoluble amorphous heme to pituitary extract (table 4, preparation 3), and the instability of hemochromogens such as globin hemochromogen in dilute acid (21) indicate that the union between the heme and the hormone is probably physical in nature. This combination of the gonadotropic hormone with the heme is believed to be the basis for the augmentation and it is suggested that the hormone is released slowly but constantly from the injection site into the blood stream and as a result the ovary is continuously under the influence of the hormone.

The augmentation by heme can be utilized in a practical way in the assay of subminimal effective amounts of pituitary gonadotropic hormone. The use of heme has has been found to be valuable in the assay of the gonadotropic hormone of the pituitary glands of small animals such as the rabbit in which one gland often does not contain sufficient hormone to stimulate the ovaries of immature rats.

The results of the augmentation experiments show that the gonadotropic hormone of human, hog and sheep pituitary glands and the FSH prepared from sheep pituitary

glands were augmented by heme. The gonadotropic hormone extract of cow pituitary glands which was not effective when given alone showed great activity when it was given with heme. The gonadotropic extract of horse pituitary glands and extracts of PMS and PU were not augmented by heme although the gonadotropic hormone was precipitated with the heme when the mixture was adjusted to PH 5. Sheep pituitary extracts, however, when precipitated in the same way gave great augmentation. It appears, therefore, that the combination of PMS and PU with the heme was such that the rate of release and absorption into the blood stream was not altered sufficiently to increase or change their effect on the ovary. The fact that PMS and PU were not augmented by heme while extracts of hog and sheep pituitary glands were may be due to a chemical difference between the gonadotropic hormones of

TABLE 4. AUGMENTATION OF GONADOTROPIC EXTRACTS OF SHEEP PITUITARY POWDER BY HEME AND OTHER SUBSTANCES

Kind of preparation	Dose		No. of rats	Av. wt. ov.
	Prep.	Augmenter		
	mg. eq.	*mg.*		*mg.*
1. SAP87[1]	100	—	3	77
2. SAP87+crys. hemin	100	4	3	76
3. SAP87+amor. heme ppt.	100	4	6	287
SAP87+amor. heme fil.	100	—	3	10
4. SAP87+heme (reduced)	100	4	3	209
5. SAP72[1]	50	—	9	42
6. SAP72+chlorophyll	50	1 cc.	2	91
7. SAP72+cyanin	50	8	3	91
8. SAP117[1]	75	—	3	38
9. SAP117+ferric gluconate	75	11	3	43

[1] Aqueous extract of sheep pituitary powder.

PMS and PU as compared to that of pituitary glands. It is well known that there is a qualitative and quantitative difference in the way in which they affect the ovary. A difference in the chemical structure of these gonadotropic substances could influence their adsorption by the heme and also the rate of release into the blood which might be the determining factor as to whether or not they would be augmented.

Extracts of sheep pituitary powder are not augmented when they are mixed and given with crystalline hemin which shows that the hemin is ineffective until it is dissolved and mixed with the hormone extract (table 4, preparations 1, 2). It should be mentioned in this connection that when hemin is dissolved in dilute basic solution, heme, the corresponding base is liberated. It is the heme, therefore, that augments the gonadotropic extracts. When the dilute basic solution of heme is adjusted to PH 5 by the addition of acid, the heme becomes insoluble and precipitates in the noncrystalline or amorphous form (22). This amorphous heme, however, when mixed with sheep pituitary extract is also effective although it is not in solution but is in the form of a suspension (table 4, preparation 3). It is essential, therefore, in order to obtain augmentation to dissolve the hemin in dilute basic solution of approximately PH 8.5, and then mix the gonadotropic extract with the solution of heme. This procedure has been used in all our work in the preparation of the heme-gonadotropic hormone solutions (14). It is to be emphasized that this procedure in which heme is liberated from hemin in dilute basic solution, or is precipitated from this solution in the insoluble amorphous form, is indispensable if augmentation is to be obtained.

It is of interest to note that reduced heme which is an effective augmenter reacts with nitrogenous substances to form hemochromogens, and in this connection the indications are that pyridine hemochromogen augments sheep pituitary extract.

The results obtained with substances other than heme in the augmentation of gonadotropic extracts of sheep pituitary powder are given in table 4. The aqueous extract when given in a dose of 50 mg. equivalent produced ovaries which had an average weight of 42 mg. as compared to 91 mg. when the extract was given with either chlorophyll or cyanin. These data show that cyanin, which is an organic copper compound, is an effective augmenter but probably no better than inorganic copper (2). Chlorophyll is related to heme in that it consists of a porphyrin combined with magnesium in place of iron. Compounds which contain a metal joined to a porphyrin such as heme, reduced heme and chlorophyll are effective in the augmentation of gonadotropic hormone extracts obtained from pituitary glands of sheep, hog, man, cow, and also the chicken (23). The combination of the metal with porphyrin is essential as neither iron nor porphyrin alone will augment gonadotropic extracts (14). The kind of organic compound combined with the metal is of great importance also as shown by the fact that ferric gluconate did not augment. The results of preliminary tests indicate that glutathione, vitamin C, cytochrome c, sodium nucleate, saffrinine, methylene blue, cresyl violet, mercurochrome, manganese chloride, nickel sulphate and cobalt nitrate do not augment the gonadotropic hormone extract of sheep pituitary powder.

SUMMARY

Human, hog, cow and sheep pituitary gonadotropic extracts and FSH made from sheep pituitary powder were augmented by heme while extracts of horse pituitary glands, PMS and PU were not augmented. The gonadotropic hormones from sheep pituitary glands, PMS and PU, regardless of whether or not they were augmented, were associated with the heme when it was precipitated. The mechanism by which heme is believed to produce the augmentation is discussed.

Metal porphyrin compounds such as heme, reduced heme and chlorophyll were found to be effective augmenters. Cyanin was also effective while ferric gluconate and several other substances did not augment.

REFERENCES

1. Fevold, H. L., F. L. Hisaw, A. Hellbaum and R. Hertz: *Am. J. Physiol.* 104: 710. 1933.
2. Fevold, H. L., F. L. Hisaw and R. O. Greep: *Am. J. Physiol.* 117: 68. 1936.
3. Maxwell, L. C.: *Am. J. Physiol.* 110: 458. 1934.
4. Deanesly, R.: *J. Endocrinol.* 1: 307. 1939.
5. Bischoff, F.: *Endocrinology* 27: 554. 1940.
6. Hellbaum, A. A.: *Proc. Soc. Exper. Biol. & Med.* 33: 568. 1936.
7. Evans, H. M., M. E. Simpson and P. R. Austin: *J. Exper. Med.* 58: 561. 1933.
8. Kraatz, C. P.: *Am. J. Physiol.* 117: 250. 1936.
9. Saunders, F. L., and H. H. Cole: *Endocrinology* 23: 302. 1938.
10. Chen, G., and H. B. Van Dyke: *J. Pharmacol. & Exper. Therap.* 62: 333. 1938.
11. Leathem, J. H., and U. Westphal: *Endocrinology* 27: 567. 1940.
12. Breneman, W. R.: *Endocrinology* 24: 488. 1939.
13. Casida, L. E.: *Proc. Soc. Exper. Biol. and Med.* 33: 570. 1936.
14. McShan, W. H., and R. K. Meyer: *Am. J. Physiol.* 119: 574. 1937.
15. Evans, J. S., L. R. Hines, J. J. Ceithaml and F. C. Koch: *Endocrinology* 26: 1012. 1940.
16. Fevold, H. L., and F. L. Hisaw: *Am. J. Physiol.* 109: 655. 1934.
17. McShan, W. H., and R. K. Meyer: *J. Biol. Chem.* 135: 473. 1940.
18. Cartland, G. F., and J. W. Nelson: *J. Biol. Chem.* 119: 59. 1937.
19. Katzman, P. A., and E. A. Doisy: *J. Biol. Chem.* 107: 513. 1934.
20. Drabkin, D. L., and J. H. Austin: *J. Biol. Chem.* 112: 91. 1935–36.
21. Anson, M. L., and A. E. Mirsky: *J. Physiol.* 60: 221. 1925.
22. Anson, M. L., and A. E. Mirsky: *J. Physiol.* 60: 50. 1925.
23. Meyer, R. K., C. H. Mellish and H. S. Kupperman: *J. Pharmacol. & Exper. Therap.* 65: 104. 1939.

MEASUREMENT OF ANTIDIURETIC ACTIVITY AS APPLIED TO ECLAMPTIC URINE AND PROPERTIES OF ANTIDIURETIC SUBSTANCES IN RAT URINE, PITUITARY AND BEEF LIVER[1]

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THE FINDING of Gilman and Goodman (1) that dehydrated rats excrete antidiuretic material in the urine has stimulated successful investigations on the presence of antidiuretic material in human urine in eclampsia (2, 3) and in other diseases with edema (4). All these workers prepared the urine for bioassay by dialysis in cellophane tubing. While this method has proved to be very useful, Walker (5) has reported that it gave a considerably lower recovery of pituitrin than the collodion adsorption method which he introduced. His method is based on the adsorption of the material when it is filtered through a collodion membrane and its subsequent elution with dilute acid. It has recently been found (6) that human urine extracts contain substances which prolong the antidiuretic effect of posterior pituitary extracts when both are injected together subcutaneously. Such augmentation, well known with hormones (6), results in erroneously high assays. Therefore, in the application of the dialysis and the collodion adsorption methods to the antidiuretic assay of human urine, it is necessary to determine whether they carry along such augmentor substances.

Gilman and Goodman presented evidence that the rat urinary material was of posterior pituitary origin. Walker, however, found that the rat urinary material was stable and pituitrin was unstable in rat urine, thereby indicating that they were different. Theobald (7) had claimed earlier that the antidiuretic material in eclamptic blood differed from the pituitary substance and suggested that it might be the same substance which he and White (8) had found in ox liver. Teel and Reid (2) reported that normal pregnant and non-pregnant women, who were dehydrated by restricting their water intake, excreted antidiuretic material in the urine. These findings suggested the possibility that the urinary material in eclampsia and other diseases might be the same as the dehydration rat and human urinary material or the liver substance and different from the pituitary substance.

In the present work we have investigated both the dialysis and collodion adsorption antidiuretic methods as applied to eclamptic urine. In addition, we have repeated the experiment of Walker which distinguished the pituitary and rat urinary substances and the work of Theobald and White on the antidiuretic material in beef liver, and have studied certain properties of these antidiuretic substances.

METHODS

Preparation of eclamptic urine for bioassay. The urine, preferably a catheterized specimen, was deproteinized by adding acetic acid and heating to 80°C. The deproteinized urine was prepared for bioassay by either the dialysis or collodion adsorption

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method. The dialysis was carried out in cellophane tubing of 0.00072 inch thickness against running tap water for 3 hours. The dialyzed urine was then slightly acidified and concentrated in vacuo to a volume of about one-tenth that of the original urine taken. With the collodion adsorption method the urine was concentrated in vacuo 5 to 10 times and treated according to the directions of Walker (5). The final volume in both preparations was adjusted so that it represented about 10 cc. urine per cc.

Bioassay. The rat method of Burn (9) was used as modified by Teel and Reid (2) Four male rats of approximately the same weight were used for an assay. Their food but not water, was removed the evening before. At the start of the assay they were placed in a metabolism cage and given a dose of warm tap water, equal to 5% of the body weight, by stomach tube. An hour later they were given a second dose of water equal to 2.5% of the body weight. Twenty minutes later 1 cc. of the preparation to be assayed was injected subcutaneously into each rat. The urine excreted from the time of the first dose of water to the injection was collected. Then the urine was collected at about 20-minute intervals and weighed. The antidiuretic activity was measured by the time required to excrete 50% of the ingested water. This quantity was taken to be one-half the difference between the water given by stomach tube and the urine excreted up to the time of the injection. Groups of 4 rats were standardized with various doses of pitressin (Parke, Davis). The activity was expressed in milliunits (mU).

Collection of rat dehydration urine. The urine of rats, deprived of both food and water, was collected after the first 24 hours. The pH of the urine was kept at about 6. The feces, caught on gauze stretched between the bottom of the cage and the collecting funnel, were removed at intervals.

Preparation of beef liver extract. The antidiuretic material from beef liver was prepared according to Theobald and White (8). Their directions consisted in the additon of 3 times the weight of water to minced ox liver, deproteinizing at pH 4.5 and 80°C., concentrating in vacuo, adjusting to pH 5, and fractionating with 75% alcohol in 2 stages. The active material was found in the alcohol filtrate and was stored this way in the cold. We have prepared two liver fractions, one from a two-stage alcohol fractionation (fraction *B*) and the other from a single alcohol fractionation (fraction *A*). Fraction *B* was prepared from a liter of the original aqueous liver filtrate which was concentrated to 107 cc., adjusted to pH 5.0 with 0.5 cc. concentrated NaOH, precipitated with 403 cc. 95% alcohol, allowed to stand over night in the refrigerator and filtered. The second alcohol filtrate was obtained by concentrating the first alcohol filtrate in vacuo to 14.5 cc. and adding 54.5 cc. of alcohol. The oily precipitate which formed was filtered off. Fraction *A* was a first alcohol filtrate that had been standing 10 days in the refrigerator. It had been similarly prepared after concentrating a liter of the same original filtrate to 132 cc.

RESULTS

Dialysis method. The dialysis method of preparation of eclamptic urine has been found to be unsuitable for quantitative antidiuretic assay by subcutaneous injection. This is shown by the augmentation obtained with pitressin when it is injected with dialyzed inactive eclamptic urine preparations (table 1). It is seen, for example, that a dose of 0.86 mU of pitressin mixed with a preparation from 8 cc. of urine gave an apparent value of 18.5 mU or an augmentation of 21.5 times. We have found only 15 and 35% recoveries of pitressin when it is dialyzed at 1 mU per cc. in 0.00072 inch cellophane, thus confirming the similar experience of Walker.

Collodion adsorption method. Evidence that the collodion adsorption method is superior to the dialysis method with respect to both augmentation and yield is given by the following experiment. The same urine and quantity which gave an augmenta-

tion of 7.4 times with the dialysis method gave only a 75% recovery of 4 mU pitressin per cc. with this method. That this recovery was unaffected by augmentation was shown by the fact that pitressin alone at the same concentration gave a 81% recovery.

Increased sensitivity of antidiuretic assay by NaCl augmentation. When we first used the collodion adsorption method with pitressin we obtained apparent recoveries of 5 to 10 times the amounts actually taken. This was found to be due to augmentation caused by the NaCl present in the eluate after neutralizing the 0.2 N HCl. Therefore we constructed a new standard curve with pitressin in 1.17% NaCl (fig. 1). It is evident that the NaCl augmentation increases the sensitivity of the bioassay to the same degree. As low as 0.8 mU of pitressin can be easily assayed with this method,

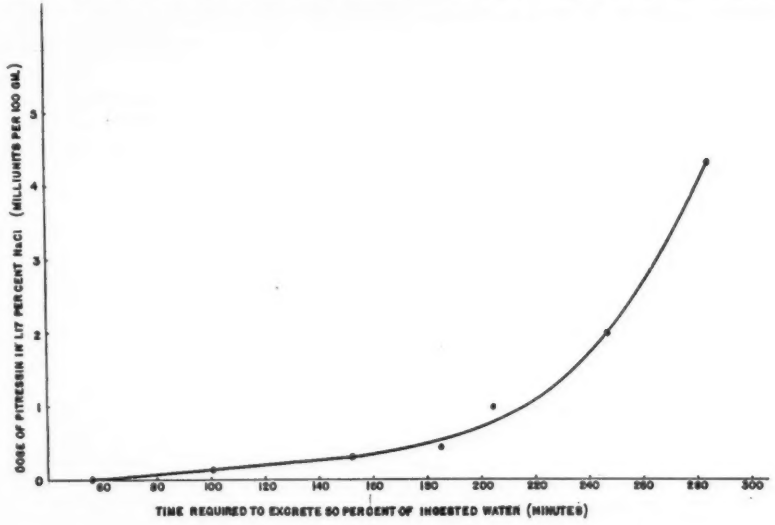

Fig. 1. STANDARD CURVE OF THE ANTIDIURETIC RESPONSE OF PITRESSIN IN 1.17% NaCl. The point at zero is the response with 1.17% NaCl alone. This curve must be restandardized at intervals.

For example, a 1.17% NaCl solution alone gave an excretion time of 56 minutes, while 0.136 mU of pitressin per 100 gm. rat gave 101 minutes. With 4 rats weighing 150 gm. each, the assay would only require a total of 0.82 mU.

Evidently Noble et al. (6) overlooked this augmentation by working with a smaller quantity of NaCl. In studying the augmentation of different doses of the pituitary antidiuretic substance with zinc acetate these authors found one case in four which gave an erratic value and concluded that zinc augmentation could not be used for a quantitative assay. With NaCl augmentation we have found no erratic results with 6 points on our standard curve with doses of pitressin between 0.136 mU per 100 gm. rat and 4.35 mU per 100 gm. rat. We did obtain an irregular result with a dose of 7.48 mU per 100 gm. rat giving an apparent value of 2.6 mU per 100 gm. rat. However, this was interpreted as probably indicating either the upper limit of accuracy of the method or an erratic point which we have encountered even in pure pitressin assays. Our highest assay with this method has been 2.34 mU per 100 gm. rat (recovery experiment) and most of our assays have been under 0.4 mU per 100 gm. rat.

Antidiuretic material in eclamptic urine. The antidiuretic material found in the same urine in three cases of eclampsia by the dialysis and collodion adsorption methods is given in table 2. It is seen that the dialysis method gave values between 1.6

TABLE 1. AUGMENTATION OF PITRESSIN BY DIALYZED INACTIVE ECLAMPTIC URINE

Urine in preparation, cc. per rat	Dose of pitressin, mU per rat	Pitressin found, mU per rat	Augmentation %
8	0.86	18.5	2150
11	1	17.9	1790
2.3	1.25	9.25	740
1	6	21.6	360

and 40 mU per 10 cc. whereas the collodion adsorption method gave values between 0.17 and 0.41 mU per 10 cc. The ratios of the two values varies considerably for the different urines. It would appear from the evidence presented above that these dialysis values are untrustworthy due to augmentation and that the collodion adsorption values more closely represent the actual activity present.

The eclamptic antidiuretic material has been obtained in a stable powder form. Deproteinized urine was evaporated in vacuo and dried with successive additions of absolute alcohol and acetone over P_2O_5 in a vacuum desiccator. A sample from 195 cc. of urine contained 6.5 mU in 1.09 gm. of powder.

TABLE 2. ANTIDIURETIC MATERIAL IN ECLAMPTIC URINE

Case	Urine specimen	Urine, sp. g.	mM/10 cc.		Ratio of dialysis to adsorption value
			Dialysis	Adsorption	
C. Intrapartum eclampsia	1	1.017	1.6	0.17	9.5
	2	1.021	40.0	0.17	235.
C.E. Intrapartum eclampsia	1	1.018	14.0	0.41	34.
T. Post partum eclampsia	1	1.025	15.0	0.33	>45.

Properties of rat urinary, pituitary and beef liver antidiuretic substances. We have investigated certain properties of the rat urinary material and compared them with those of the pituitary substance and the liver substance of Theobald and White. Unless otherwise mentioned all the assays in the following work were made by the collodion adsorption method. Walker reported that the rat dehydration material was stable when it was allowed to stand for 24 hours in rat urine at pH 6.6 to 7.4, but that the pituitary antidiuretic substance was destroyed under the same conditions. We have found that in two such experiments pitressin at a concentration of 4 mU per cc. of rat urine at pH 7.0 was destroyed 87 and 99%. In a third experiment, pitressin at 4 mU per cc. was added to rat dehydration urine. The rat urine alone contained 0.48 mU per cc. of activity after standing. Only 0.18 mU per cc. of activity was found in the urine containing pitressin after standing. Thus the destruction of pituitrin and the relative stability of the rat urinary material in rat urine is indicated by these experiments.

The two liver fractions (described above), *B*, from a 2-stage alcohol fractionation and, *A*, from a single stage alcohol fractionation, were assayed after removing the alcohol in vacuo. Fraction *B* was inactive. Analysis of fraction *A* by the dialysis method indicated an antidiuretic content corresponding to 230 mU per 100 gm. of liver, whereas the collodion adsorption method gave only 14 mU per 100 gm. of liver. Evidently the activity in fraction *B* had been lost by concentrating the first alcohol filtrate too far. A sample of fraction *A* was dialyzed and then analyzed by the collodion adsorption method. Six mU per 100 gm. of liver were found, therefore, 57% of the activity was lost by dialysis. Thus the liver substance behaved like the other antidiuretic substances with respect to cellophane dialysis and collodion adsorption.

Theobald and White treated their liver extract with HNO_2 to remove histamime and found that the antidiuretic activity was increased. We have confirmed this finding (table 3); 0.9 mU of liver substance in 5 cc. was treated for 1 hour in an ice bath with HNO_2, then being removed from the bath was allowed to stand 3 hours at room temperature. After this treatment the liver substance increased to 1.9 mU or 110%. The N_2O_3 was generated by passing concentrated H_2SO_4 into a 20% $NaNO_2$ solution. The rat dehydration and pituitary substances were similarly treated with HNO_2 (table 3). The fact that the rat dehydration activity is likewise increased (180%) and the pituitary activity is almost completely destroyed (86%), suggests that in HNO_2 treatment we may have another means of distinguishing these antidiuretic substances and that the liver substance may be the same as the rat urinary substance.

TABLE 3. EFFECT OF NITROUS ACID ON ANTIDIURETIC SUBSTANCES

Substance	Before treatment mU/5 cc.	After treatment mU/5 cc.	Change, %
Liver	0.9	1.9	+110
Rat dehydration	1.1	3.1	+180
Pitressin	10.0	1.4	− 86

Theobald and White reported that the liver substance was destroyed by boiling, but did not specify the conditions. This property has been cited (10) as distinguishing it from the pituitary substance. Walker, on the other hand, has found that the rat dehydration and pituitary substances have a similar resistance to boiling. It is known that the pituitary antidiuretic substance is partially destroyed by boiling (11). We have determined that the pituitary substance at 1 mU per cc. is 72% destroyed when boiled for 10 minutes in neutral solution. With smaller concentrations of the liver and rat urinary substances, we found that both are partially resistant to boiling under these conditions. Therefore, when these antidiuretic substances are compared under the same conditions of boiling, they are found to behave similarly.

DISCUSSION

Our experiments with the dialysis and collodion adsorption methods as applied to the assay of the eclamptic urinary antidiuretic substance by subcutaneous injection indicate that only the latter method is suitable for quantitative purposes. It has been shown that dialyzed inactive eclamptic urine preparations contain substances which augment the antidiuretic activity of pitressin. This augmentation is eliminated when the material is injected intravenously (6), but such injections in rats (tail vein (3)) are not convenient. We have not studied the augmentation with intramuscular or intraperitoneal injections, but we should expect it to occur. The dialysis method also gives lower recoveries with pitressin and has been reported (4) to give toxic urine preparations. In the present work no toxicity has been noticed with either method.

It should be pointed out that neither the dialysis nor the collodion adsorption method has been studied for its yield with known amounts of the rat or human urinary antidiuretic substance. Walker has found 45% less of the rat urinary substance with the collodion adsorption method than with the dialysis method, but the assays with the latter method may have been affected by augmentation.

The use of NaCl augmentation to increase the sensitivity of the antidiuretic assay by subcutaneous injection is another advantage of the collodion adsorption method. In this case the augmentation is controlled, whereas it is not with dialyzed urine preparations.

From a clinical point of view our finding of the eclamptic urinary substance in concentrations of only 0.17 to 0.41 MU per 10 cc. by the collodion adsorption method, in contrast to 1.6 to 40 MU per 10 cc. by the dialysis method, greatly reduces the significance of the material in producing possible pathological effects. Previous workers (2) using the dialysis method have reported that eclamptic urines contained 35 to greater than 100 MU per 10 cc. The urinary antidiuretic substance has been associated with edema and oliguria in eclampsia and other diseases (2, 3, 4). None of our cases had edema and only one (*C.E.*) had an oliguria.

The experiments on the instability of pitressin and the relative stability of the rat urinary substance support the contention of Walker that they are different. Further support is given by the different behavior of these substances toward nitrous acid. The similar behavior of the liver and rat urinary substances to dialysis, collodion adsorption, boiling and especially to nitrous acid suggests that they may be identical. Theobald claimed that the eclamptic blood and beef liver substances could be distinguished from the pituitary substance, infundin, by their property of passing through a collodion membrane. We have found no such difference with our membrane.

Other known antidiuretic substances of the liver are a contaminant of a heparin preparation (12) and histamine (8), but both are distinguished from the present liver substance in being inactive by subcutaneous injection.

SUMMARY

When pitressin together with dialyzed inactive ecalmptic urine was assayed for antidiuretic activity by subcutaneous injection in rats, amounts up to 21.5 times the pitressin activity were found. An inactive eclamptic urine which gave an augmentation of 7.4 times the pitressin activity with the dialysis method, gave only a 75% yield when added to pitressin and assayed by the collodion adsorption method. The antidiuretic activity in the urine of three cases of eclampsia was 1.6 to 40 MU per 10 cc. with the dialysis method and only 0.17 to 0.41 MU per 10 cc. with the collodion adsorption method, the difference being attributed to augmentation.

The sodium chloride in the eluate in the collodion adsorption method augmented pitressin activity 5 to 10 times. Using this augmentation to increase the sensitivity of the antidiuretic assay as little as 0.8 MU of pitressin may be assayed.

The destruction of pitressin and the relative stability of the rat urinary substance in rat urine was indicated. The occurrence of an antidiuretic substance in beef liver was confirmed. The liver substance behaved like the eclamptic, pituitary and rat urinary substances with respect to cellophane dialysis and collodion adsorption, like the latter two substances with respect to boiling, and like the rat urinary substance, but unlike the pituitary substance, with respect to treatment with nitrous acid.

REFERENCES

1. GILMAN, A., AND L. GOODMAN: *J. Physiol.* 90: 113. 1937.
2. TEEL, H. M., AND D. E. REID: *Endocrinology* 24: 297. 1939.
3. KRIEGER, V. I., AND T. B. KILVINGTON: *Med. J. Australia* 1: 575. 1940.
4. ROBINSON, H. P., JR., AND L. E. FARR: *Ann. Int. Med.* 14: 42. 1940.
5. WALKER, A. M.: *Am. J. Physiol.* 127: 519. 1939.
6. NOBLE, R. L., H. RINDERKNECKT AND P. C. WILLIAMS: *J. Physiol.* 96: 293. 1939.
7. THEOBALD, G. W.: *Clin. Science* 1: 225. 1934.
8. THEOBALD, G. W., AND M. WHITE: *J. Physiol.* 78: 18P. 1933.
9. BURN, J. H.: *Quart. J. Pharm. & Pharmacol.* 4: 517. 1931. Biological Standardization. Oxford Univ. Press 1937. p. 69.
10. VAN DYKE, H. B.: Physiology and Pharmacology of the Pituitary Body. University of Chicago Press, 1936, p. 335.
11. HELLER, H.: *J. Physiol.* 96: 337. 1939.
12. WALKER, A. M.: *Proc. Soc. Exper. Biol. & Med.* 39: 105. 1938.

COMPARISON OF ASSAY METHODS USING INTERNATIONAL STANDARD LACTOGEN[1,2]

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IN A PREVIOUS COMMUNICATION (1) a comparison was presented of a mammalian and several pigeon assay methods for the lactogenic hormone. Since completing that work, the International Standard Lactogenic hormone became available (2). Lyons (3) has recently reported that 80 I.U. are required to induce lactation in 50% of 20 estrous guinea pigs weighing from 650 to 1000 gm. Since we (4) are convinced that the factor initiating lactation in mammals is identical with that stimulating the pigeon crop gland and since the pigeon crop gland is extremely sensitive to the lactogenic hormone, it seemed desirable to determine the amounts of the International Standard required to produce a unit response by various modes of administration in the pigeon which are used in routine assays in our laboratory.

The three pigeon assay methods used were the same except for the route of injection; namely, *a*), shallow intrapectoral (5), *b*), subcutaneous over the pectoral muscles and *c*), intradermal over each crop gland (micro test). The unit for the assays is defined as the total amount of hormone injected during a period of 4 days which will cause a minimum but definite stimulation of the crop glands of $50 \pm 11\%$ of 20 common pigeons weighing 300 ± 40 gm. The crop glands in all cases were examined by two observers using transmitted light.

EXPERIMENTAL

It was observed that 0.1 mg. of the I. S. lactogen would cause a response within the unit range when the subcutaneous route of injection was used (table 1). Thus, 3 groups of pigeons injected at this dosage level gave a 45%, 55% and 55% response respectively. When 0.11 mg. of I. S. lactogen were injected subcutaneously a 70% response resulted, whereas 0.125 mg. and 0.15 mg. gave a 76% and 80% response respectively. The shallow intrapectoral method (McShan-Turner) of injection required 0.125 mg. of I. S. lactogen for a response within the unit range, 25% more hormone than was required by the subcutaneous method. The intradermal (micro) method of injection over the crop gland required only 0.000624 mg. of I. S. lactogen for a response within the unit range. Five groups of birds were injected at this dosage level and the response in each case was from 45% to 55% positive. It will be noted in the case of each method used that the results were consistent and reproducible at the unit dosage level with successive groups of pigeons.

A comparison of various lactogenic assay methods in terms of the I.U. is presented in table 2. These comparisons, with the exception of the Riddle-Bates pigeon unit, and Lyons pigeon and guinea pig units, were determined on the basis of our previous work on the comparison of assay methods of the lactogenic hormone (1). The I. S.

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lactogen is reported to contain 10 Riddle-Bates U per mg. or one Riddle-Bates U per 0.1 mg. Since our subcutaneous unit also requires 0.1 mg. of I. S. lactogen, it is obvious that the Riddle-Bates unit, the I.U. and our subcutaneous unit are all equal. Our shallow intrapectoral unit is equal to 0.80 of a subcutaneous or I.U. This unit is 20% less sensitive than the subcutaneous unit. Our intradermal (micro) unit is 160 times more sensitive than the subcutaneous unit, whereas the Reece-Turner intradermal unit is only 22.2 times more sensitive than the subcutaneous unit. On the basis of a private communication already reported, we deduce that Lyon's pigeon unit is equal to 0.60 of an I.U. According to Lyons (3) his guinea pig unit is equal to 80 I.U. The Gardner-Turner rabbit response requires approximately 340 I.U.

In connection with the intradermal method of injection, we did not find that differences in injection volumes containing the same amount of hormone would cause

TABLE 1. COMPARISON OF CROP GLAND ASSAYS USING I. S. LACTOGEN (1 I.U. = .1 mg.)

No. pigeons in group	Amt. of I.S. lactogen injected per pigeon	Injection route	Total volume injected	Positive response	Percentage response	
	mg.		*cc.*			
10	0.15	Subcutaneous	0.40	8	80	
30	0.125		0.40	23	76	
20	0.11		0.40	14	70	
20	0.10		0.40	9	45	Unit range
20	0.10		0.40	11	55	Unit range
20	0.10		0.40	11	55	Unit range
20	0.125	Shallow	0.40	10	50	Unit range
20	0.125	intrapectoral	0.40	11	55	Unit range
10	0.000416	Intradermal	0.40	0	0	
10	0.000520	(micro)	0.40	0	0	
10	0.000624		0.40	5	50	Unit range
20	0.000624		0.20	9	45	Unit range
20	0.000624		0.40	11	55	Unit range
20	0.000624		0.60	9	45	Unit range
10	0.000624		1.00	5	50	Unit range
10	0.000832		0.40	10	100	

any significant variation in the response of the crop gland, as was recently reported by Bates and Riddle (6). This question was of interest to us inasmuch as we sometimes inject different volumes intradermally when assaying blood or urinary extracts. The results given on the intradermal test in table 1 show that 2, 3 and 5-fold differences in injection volume (0.2 cc., 0.4 cc., 0.6 cc., and 1.0 cc.) containing the same amount of hormone failed to cause a significant difference in the response of the crop gland. The differences in crop gland response which Bates and Riddle (6) noted may be due to the fact that they made only 2 injections over a 2-day period whereas our birds were injected 4 times over a 4-day period.

DISCUSSION

The availability of I. S. lactogen, and the acceptance of the I.U. of lactogen as a standard, enables one to compare the various lactogenic assay units developed in different laboratories. The present study is believed to contribute to an understanding of the quantitative relationship between these lactogenic units.

Each of the methods used have their advantages. Thus, although a mammal may be preferable for assaying lactogen, it is obvious that such a method requires considerable quantities of hormone. The pigeon, on the other hand, is much more sensitive

to lactogen; also, the cost of pigeons is less and they do not require any preliminary preparation, i.e., growth of the mammary gland.

The advantages of the pigeon assay methods presented in this paper are as follows. (*a*), Common pigeons of both sexes can be used. These pigeons are always plentiful, thus eliminating the more expensive breeding or purchasing of special breeds. (*b*), The tests are all based on the sensitive 50% minimum crop proliferation response, which we believe to be more accurate than the crop weight method. (*c*), All three tests are strictly comparable, and repeatable with successive groups of birds. (*d*), The two systemic tests are particularly applicable when large quantities of lactogenic preparations are available for assay. The subcutaneous unit has the added advantage of being equal to the I.U. (*e*), The highly sensitive intradermal (micro) unit, which requires

TABLE 2. COMPARISON OF LACTOGENIC ASSAY UNITS

Assay unit	Assay animal	No. of assay units per I.U.		No. of I.U. per assay unit	
Subcutaneous unit	Common pigeon	1.00	=1.00	1.00	=1.00
Shallow intrapectoral unit		0.80	=1.00	1.25	=1.00
Intradermal unit (micro test)		160.00	=1.00	0.00625	=1.00
Reece-Turner intradermal unit	Common pigeon	22.2	=1.00	0.045	=1.00
Riddle-Bates intrapectoral unit	Specific strains of doves and pigeons	1.00	=1.00	1.00	=1.00
Lyons subcutaneous unit	Silver King squabs	0.60	=1.00	1.66	=1.00
Gardner-Turner rabbit unit	Mature female New Zealand White Rabbits	0.00294	=1.00	340.00	=1.00
Lyons guinea pig unit	Nulliparous female guinea pigs weighing 650–1000 gm.	0.0125	=1.00	80.00	=1.00

so little hormone, is advantageously used to assay the extent of lactogenic contamination in other purified pituitary preparations. It is the only practical method available to assay the lactogenic content of blood, urine and other body tissues.

SUMMARY

Three methods of assay of I. S. lactogen have been compared. All depend upon a 50% minimum crop gland proliferation response in 20 common pigeons, weighing 300 ± 40 gm. It was found that by the subcutaneous route of administration 0.1 mg. of the I. S. was required which is equal to the I.U.; that the shallow intrapectoral method requires 1.25 I.U.; and that the intradermal (micro) unit requires 1/160 of an I.U. The comparative relation of various other lactogenic assay units to the I.U. was given. In connection with the intradermal unit, it was shown that 2, 3 and 5-fold differences in injection volume containing the same amount of hormone caused no change in effectiveness of the crop gland response.

REFERENCES

1. BERGMAN, A. J., J. MEITES AND C. W. TURNER: *Endocrinology* 26: 716. 1940.
2. Report of the Third International Conference on the Standardization of Hormones. Geneva, Aug. 11–13, 1938. *Bull. Health Organization League of Nations*, Vol. VII, Ext. No. 32. Emmens, C. W.: *Bull. Health Organization League of Nations*, Vol. VIII, Ext. No. 19.
3. LYONS, W. R.: *Anat. Rec. Supp.* 2, 76: 39. 1940.
4. BERGMAN, A. J., AND C. W. TURNER: *J. Dairy Sci.* 1940.
5. MCSHAN, W. H., AND C. W. TURNER: *Proc. Soc. Exper. Biol. & Med.* 34: 50. 1936.
6. BATES, R. W., AND O. RIDDLE: *Proc. Soc. Exper. Biol. & Med.* 44: 505. 1940.

INFLUENCE OF EPINEPHRIN AND ADRENAL CORTICAL EXTRACT ON THE LACTOGENIC PROPERTY OF PROLACTIN

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CLINICAL APPLICATION of the lactogenic hormone from the anterior pituitary gland has been successful in the treatment of deficient lactation in human beings. Kurzrok, Bates, Riddle and Miller (1) reported that doses of 75 to 400 U of prolactin were capable of significantly increasing milk production in 25 of a group of 29 women who had failed to lactate adequately up to the 16th day postpartum. Similar results have been described (2, 3). This lactogenic effect of the hormone is consistent with the clear-cut findings observed in laboratory animals under experimental conditions (4, 5). Stewart and Pratt (6), on the other hand, reported that prolactin in doses up to 3000 U from the 6th to 9th post-partum days failed to show any significant influence on the secretory rate in 14 women with deficient lactation as compared with a group of 10 patients receiving a placebo treatment. The secretory rate in both groups increased by approximately 40%. They concluded that the action of prolactin in animals and women is not analogous.

Negative or disappointing results from such medication in the human postpartum period may be explained in one or both of two ways. The lactogenic hormone may be unable uniformly to initiate or significantly influence lactation in certain human subjects because the endocrine mechanism differs fundamentally from that observed in experimental animals, or because it is subnormal in certain respects. Lactation during the postpartum period is very probably dependent on the activity of several interrelated glands of internal secretion. Thus, the absence of therapeutic response to prolactin may well be due to subnormal functioning of one or more glands, leading to a deficient supply of one or more of the hormones which together are required to establish that concatenation of physiological circumstances essential for lactation.

The second of these two possibilities offers the more fruitful field for investigation. In this connection, Reece (7) has studied the effect of the influence of prolactin supplemented with adrenal cortical hormone on the lactation of the pseudopregnant albino rat. He concluded that the latter hormone in doses of 15 to 24 dog units, particularly when further supplemented with a 20% glucose solution, produces a higher order of lactation when administered together with 100 to 150 U of prolactin than can be obtained by the administration of the same dose of prolactin alone. Reece's work takes on added significance when it is correlated with the findings of Folley and Young (8), who found that prolactin preparations high in glycotropic factor were particularly effective in stimulating bovine lactation. More recently, both Hartman and his coworkers (9) and Grattan and Jensen (10) have pointed out the anti-insulin, or glyconeogenetic effect of adrenal cortical extracts. It may well be that the augmentation of the lactogenic effect of prolactin by cortical extracts, as demonstrated by Reece, is the result of a physiological hyperglycemia. It was with these findings in

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mind that we decided to investigate the effect of a continued hyperglycemia on the prolactin initiated lactation in the pseudopregnant rat.

While hyperglycemia results from the parenteral administration of aqueous solutions of epinephrin, it is very transient and unsatisfactory for the purpose desired, i.e., a prolonged elevation of the blood sugar level. This type of reaction was obtained by employing a 1:2500 solution of suprarenin bitartrate in 16% gelatin. Such solutions, when injected subcutaneously, are practically free from local reactions, and due to the depot effect, produce prolonged epinephrin action. This effect is apparent in table 1, which indicates the blood sugar levels of a series of rats following such an injection. These animals were similar in all respects to those of our experimental series and were maintained under the same conditions. Blood samples were obtained from the tail vein and were added to a small amount of powdered potassium oxalate. Blood sugar levels were determined by the (micro) Folin-Malmros method.

Our experimental animals consisted of a series of adult female albino rats weighing

TABLE 1. EFFECT OF THE SUBCUTANEOUS ADMINISTRATION OF 1:2500 EPINEPHRIN IN 16% GELATIN ON THE BLOOD SUGAR OF ADULT FEMALE RATS

Rat	Dose	Blood sugar (mg./100 cc.) Hours after injection					
		0	1	2½	3	5	7
	cc.						
A	0.1	117	—	166	—	165	—
D	0.2	114	—	200	—	153	—
E	0.2	133	190	—	170	152	119
H	0.2	128	202	214	—	128	—
I	0.2	147	174	175	—	142	—
J	0.3	129	183	196	—	152	—
K	0.3	107	175	144	—	130	—

from 250 to 300 gm., taken from our laboratory colony. These animals had been isolated from males for a period of 5 weeks prior to initial experimental procedures.

Pseudopregnancy was induced by the injection of 100 I.U. of korotrin (chorionic gonadotrophin obtained from human pregnancy urine) given in 6 divided doses over a period of 2 days. On the sixth day after the first korotrin injection the animals were ovariectomized and one mammary gland was removed as a control to indicate the state of activity of the mammary tissue prior to further medication. Immediately after ovariectomy the animals were divided into small groups and treated according to the schedule described in table 2. The concentrations of the endocrine preparations (other than epinephrin) were so adjusted that the required quantity for any single injection was contained in 0.25 cc. The entire dosage was given in 6 injections administered subcutaneously at equally spaced intervals during the 2 days following ovariectomy.

Prolactin was prepared according to the method of Bates and Riddle (11) and standardized on Carneau pigeons against the international prolactin standard: the preparation contained 10.8 U per mg. An adrenal cortex extract of proven potency was obtained from Wilson and Company: 1 cc. was equivalent to 75 gm. of fresh tissue. We have found this material to be at least as potent, if not more so, than eschatin, the material employed by Reece. D'Amour has reported the two preparations to be of the same potency (12). The epinephrin in gelatin solution was similar to that described by Spain et al. (13) and fundamentally consisted of a 1:2500 solution of suprarenin bitartrate in 16% gelatin. This material had to be warmed to 30°C. before it could be injected.

All animals were killed 4 hours after the last injection and mammary tissue was removed for examination. The results are shown in table 2.

Histological rating of mammary activity

(−) Negative.

(± to +) Occasional dilated acinus containing secretion. Major portion of epithelium in resting columnar form.

(++) Secretory activity manifested by partial flattening of epithelial cells and partial dilatation of acini.

(+++) Maximal activity evidenced by marked flattening of epithelial cells, gross dilatation of acini, and presence of secretion mass in ducts.

The control mammary tissue was removed from each animal at the time of ovariectomy and prior to the administration of any specific lactogenic material. All such

TABLE 2

Group	No. of animals	Medication			Mammary activity—Histological				Incidence of frank lactation
		Prolactin	Epinephrin	Cortical extract	−	± to +	++	+++	
		I.U.	cc.	cc.	%	%	%	%	%
A	10	0	0	0	20	80	0	0	0
B	15	50	0	0	0	20	60	20	20
C	6	100	0	0	0	17	33	50	50
D	11	150	0	0	0	9	27	64	54
E	15	50	0.6	0	0	0	20	80	40
F	17	50	1.2	0	0	0	12	88	70
G	5	100	0.6	0	0	20	40	40	40
H	4	150	0.6	0	0	0	25	75	75
I	5	100	1.2	0	0	0	60	40	100
J	5	0	0	1.0	20	80	0	0	0
K	15	50	0	0.6	0	7	46	47	0
L	15	50	0	1.0	0	0	66	34	13
M	12	100	0	0.6	0	0	58	42	67
N	13	150	0	1.0	0	23	15	62	46
O	5	100	1.2	0.6	0	20	0	80	60
P	5	0	0.6	0	40	60	0	0	0

tissues, although grossly inactive, showed slight, but definite, indications of a secretory activity when examined microscopically. The same order of secretory activity was observed in the untreated control series.

The administration of prolactin induced a grossly apparent lactation in approximately 50% of the animals in the series; increasing the dose from 50 to 100 U had a marked effect on the activity of the mammary gland, but a further increase in dosage had no significant effect.

The simultaneous administration of epinephrin with prolactin caused a significant increase in the incidence of frank lactation: *group I*, all of the experimental animals showed frank lactation following the administration of 100 U of prolactin and 0.2 cc. of a 1:2500 solution of epinephrin in 16% gelatin. This effect is only slightly greater than that resulting from the administration of 0.6 cc. of cortical extract together with 100 U of prolactin. However, when a minimally effective dose of prolactin (50 I.U.) was administered, the augmentation of the lactogenic effect by epinephrin was much

more marked than that produced by the simultaneous administration of cortical extract. The administration of epinephrin alone has no lactogenic action.

The addition of cortical extract to the combined epinephrin+prolactin regime did not enhance the effect produced.

DISCUSSION

Our data support two of Reece's major conclusions since we find that secretory activity is induced in the mammary glands of a certain number of control animals, and that the simultaneous administration of prolactin and adrenal cortical hormone can initiate lactation of a somewhat higher order than that induced by the same dose of prolactin alone. This effect cannot be attributed to 'cortilactin' since injections of the cortical extract alone produce no greater degree of mammary activity than that observed in the controls.

Our results indicate, however, that secretory activity of a very high order can be induced by the administration of prolactin and epinephrin in 16% gelatin. In our experimental series, the combination prolactin+epinephrin produced a better result than the combination prolactin+adrenal cortical extract. Reece apparently also considered the idea that hyperglycemia might have a favorable effect, when he supplemented his prolactin and cortical hormone with injections of 20% glucose. However, no augmentation of lactogenic effect due to the latter treatment is apparent in his rating of the mammary glands. This is in accord with our findings that the combination prolactin+adrenal cortical extract+epinephrin is no more effective than the combination prolactin+epinephrin.

Several recent publications (14, 15) have called attention to the potentiation of anterior pituitary hormones when injected simultaneously into the same site as epinephrin. This potentiation is attributed to the vasoconstrictor action of epinephrin and its resultant diminution of the vascularity of the area containing the injected hormone, thus slowing down the absorption of the hormone. This mechanism cannot explain the results obtained in our experiments for the injection sites were on opposite sides of the body when these two hormones were administered. It is our opinion that the potentiation of the lactogenic properties of prolactin by epinephrin is a result of the hyperglycemia resulting from the slowly absorbed epinephrin. A similar state of hyperglycemia induced in control animals is without effect.

SUMMARY

A series of 158 female albino rats has been rendered pseudopregnant by the administration of chorionic gonadotrophin. A slight degree of secretory activity is induced in the mammary glands by this treatment. The effect may be considerably augmented by the administration of 50 I.U. of prolactin, and a somewhat greater effect is produced by 100 I.U., but no significant increment of glandular activity is effected by raising the dose to 150 I.U. The effect obtained by the administration of 50 I.U. of prolactin is somewhat augmented by the simultaneous administration of adrenal cortical extract; however, a considerably greater effect is obtained if the prolactin treatment is accompanied by an epinephrin-induced state of hyperglycemia. Peculiarly, supplementation by both cortical extract and epinephrin does not seem to produce as great as that obtained by supplementation with epinephrin alone. The effect of the cortical extract may possibly be explained on the basis of its anti-insulin properties. We agree with Reece that the refractoriness of the mammary glands of the pseudopregnant rat to lactogen can be fully explained on the assumption that another hormone becomes the limiting factor before lactogen can fully express itself.

REFERENCES

1. KURZROK, R., R. W. BATES, O. RIDDLE AND E. G. MILLER: *Endocrinology* 18: 18. 1934.
2. ROSS, J. R.: *Endocrinology* 22: 429. 1938.
3. KENNY, A., AND A. KING: *Lancet* 1: 828. 1939.
4. RIDDLE, O., R. W. BATES AND S. W. DYKSHORN: *Proc. Soc. Exper. Biol. & Med.* 29: 1211. 1932.
5. LYONS, W. R., AND H. R. CATCHPOLE: *Proc. Soc. Exper. Biol. & Med.* 31: 299. 1923.
6. STEWART, H. L., AND J. P. PRATT: *Endocrinology* 25: 347. 1939.
7. REECE, R. P.: *Proc. Soc. Exper. Biol. & Med.* 40: .25. 1939.
8. FOLLEY, S. J., AND F. G. YOUNG: *Proc. Roy. Soc. s. B.* 126: 45. 1938.
9. HARTMAN, F. A., K. A. BROWNELL, R. WALTHER AND A. EDELMANN: *Endocrinology* 27: 642. 1940.
10. GRATTAN, J. F., AND H. JENSEN: *J. Biol. Chem.* 135: 511. 1940.
11. BATES, R. W., AND O. RIDDLE: *J. Pharmacol. & Exper. Therap.* 35: 365. 1935.
12. D'AMOUR, F. E.: *Endocrinology* 26: 92. 1940.
13. SPAIN, W. S., M. B. STRAUSS AND A. M. FUCHS: *J. Allergy* 10: 209. 1939.
14. EVANS, J. S., L. R. HINES, J. J. CEITHAML AND F. C. KOCH: *Endocrinology* 26: 1012. 1940.
15. FRIEDGOOD, H. B., S. BEVIN AND U. U. UOTILA: *Am. J. Physiol.* 129: 724. 1940.

THE EFFECT OF HYPOPHYSECTOMY UPON HYPERCHOLESTEROLEMIA OF DOGS[1]

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THE OCCURRENCE of hypercholesterolemia following surgical or spontaneous hypothyroidism is a well recognized fact but the mechanism by which it is produced has never been clarified.

There exist abundant clinical and experimental data which show that after thyroidectomy the hypercholesterolemia develops slowly and reaches its greatest intensity at the time the clinical signs and lowered metabolic rate also indicate a severe degree of thyroid insufficiency (1–4). The administration of thyroid brings about a gradual reduction in the level of plasma cholesterol and at the same time there is an amelioration of the other signs of deficiency.

The association of hypercholesterolemia with a lowered basal metabolic rate has led to attempts to use the former as a measure of thyroid insufficiency (5). These efforts have not been particularly successful, and, indeed, there are certain facts regarding the occurrence of hypercholesterolemia that suggest that a different mechanism than change in metabolic rate is responsible for its appearance after thyroidectomy.

In the first place calorigenic agents other than thyroid, although elevating the metabolic rate, neither alleviate the symptoms of myxedema (6) nor reduce the plasma cholesterol values. Secondly, hypophysectomy, which produces almost as great a depression of the basal metabolic rate as does thyroidectomy, is not accompanied by an elevation of the plasma cholesterol. Lastly, the plasma cholesterol is unchanged in most patients with chromophobe adenomata (space occupying tumors) of the hypophysis and decreased basal metabolic rates (7, 8).

The lowered basal metabolic rate of hypophysectomized animals is thought to be due to the associated thyroid atrophy (9), but the absence of an elevated plasma cholesterol in these animals strongly suggests that the presence of the pituitary is necessary for the appearance of hypercholesterolemia after thyroidectomy. The latter procedure is prone to produce cytological alterations in the anterior pituitary, where, when the deficiency is complete, there occurs a degranulation of the acidophils and an accumulation of colloid-like material in the basophils (10).

These facts suggest that the hypercholesterolemia which follows thyroidectomy depends on the presence of the anterior pituitary and that this gland plays a previously unsuspected active role in its occurrence.

EXPERIMENTAL

The following experiments were devised for a study of this relationship.

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Hypercholesterolemia was produced in dogs by total thyroidectomy, and later, hypophysectomy, or total adrenalectomy, was performed. The subsequent changes in the plasma cholesterol were followed throughout the experiments. Certain studies were also made in dogs whose plasma cholesterol was elevated by the injection of anterior pituitary extracts.

The dogs used were healthy, well nourished mongrels, weighing 12 to 15 kg. There were 9 males and 10 females. Each animal was fed ad libitum once daily a ration which consisted in approximately 7 parts of Baloration and one part of a canned meat preparation; once weekly a portion of 150 gm. of beef suet was added. For a few days following hypophysectomy, when swallowing was difficult, the animals were given milk to drink until they would eat the solid food. After adrenalectomy animals were fed a special diet[2] (9) to which was added 6 gm. of sodium citrate each day, and these animals were given 0.9% NaCl solution to drink instead of tap water.

Operative procedures. The anesthesia for all operations was nembutal administered intravenously or intraperitoneally, in a dosage of 30 to 35 mg. per kg.

The thyroidectomy of each dog was performed in such a way as to preserve the parathyroid at the upper pole of each lobe of the thyroid. Other parathyroid glandules, when identified, were implanted into a slit in one sternomastoid muscle.

The hypophysectomies were carried out by a simplified transbuccal approach, which permitted an extirpation of practically all of the gland, but admittedly may have allowed in a few instances a portion of anterior lobe to remain as a cuff at the base of the pituitary stalk.

The adrenalectomies of each animal were performed in sequence so that the right adrenal, the most difficult technically, was removed first. Later, when the animal's condition was good the remaining adrenal was removed.

Each operation upon the hypophysis or adrenal was followed by an intravenous infusion of 300 cc. of 10% glucose in physiological saline solution; later, in case the animal did not eat, this was repeated.

Chemical procedures. The value of the cholesterol in specimens of blood plasma of the fasted animals was determined by the method of Bloor with Sackett's modification (11, 12). The analyses were made routinely in duplicate and the mean value was recorded. Repeated examinations of portions of one sample of blood varied approximately 15%.

The calcium determinations were carried out by the Tisdall modification (13) of the method of Kramer and Tisdall. The serum protein analyses were made by the usual macro Kjeldahl method. The inorganic phosphorus content of the serum was determined by the method of Kuttner and Cohen (14). The fatty acid content of the blood serum was determined by the Stoddard and Drury method as modified by Man and Gildea (15).

RESULTS

The presumably normal preoperative plasma cholesterol values varied considerably in the 19 dogs studied. The quantities ranged from a minimum of 108 mg.% to 255 mg. %, and in two instances the value was as high as 320 mg. %. So far as could be told all were normal save one, (348 ♀) with the high value of 320 mg. %, which was in estrus at the time the first reading was taken. The others with values over 255 mg.

[2] The composition of this low-potassium diet was as follows.

⅓ white cane sugar	1/12 butter
½ casein, potassium free	1/12 crisco
⅙ agar agar	1% cod liver oil

To this stock was added small amounts of boiled canned or fresh meat, and when the experiments were long, bone and yeast were given.

% were females, and they were first studied in May and June, when without our knowledge, they may have been in or near a period of estrus.

Thyroidectomy always caused an increase in the amount of the plasma cholesterol, which, however, was sustained at high levels for many months in only 9 animals. The

TABLE 1

A. Thyroidectomy and hypophysectomy

Dog No. and sex	Plasma cholesterol, mg. %									Notes
	Before thyroidectomy	Weeks after thyroidectomy				Weeks after hypophysectomy				
		4	8	12	16	2	4	8	12	
300♀	192	305	355	—	—	178	99	—	—	Died 28 days postoperative; embolism, cardiac worms.
317♂	141	306	278	269	—	189	130	164	109	Prolonged tetany after hypox., low Ca values; improvement with Ca therapy
271♂	—	—	—	—	347	184	80	45	—	Prolonged tetany after hypox., died in convulsions 2 mo. p.o.
316♀	153	273	260	295	440	200	105	115	126	Mild tetany after hypox., improved with Ca therapy; no pit. remnant.
326♀	143	255	290	330	415	285	180	166	215	No tetany; all Ca and P values normal; no pit. remnant, but stalk hypertrophied.
348♀	320	375	385	—	310	270	270	280	320	Developed estrus p.o.; pituitary remnant found at autopsy.

B. Thyroidectomy and bilateral adrenalectomy

Dog No. and sex	Plasma cholesterol, mg. %									Notes
	Before thyroidectomy	Weeks after thyroidectomy				Weeks after 2nd Adrex.				
		4	8	12	16	1	2	4	12	
352♀	153	215	345	315	485	210	—	—	—	Ate poorly after adrenalectomy; died pneumonia 7th day.
355♀	185	235	345	380	470	130	80	—	—	Ate poorly; did badly despite cortin therapy; killed on 18th day.
379♂	215	440	400	430	—	345	420	305	265	Took diet well; condition good. Died of adr. insufficiency after high potassium diet.

failure of 7 dogs to develop a satisfactory lasting hypercholesterolemia after thyroidectomy was never explained but presumably was due to the presence of thyroid rests. At the autopsies of these 7 animals we were not able to demonstrate in the gross any such thyroid remnants. In an effort to remove all thyroid rests 2 of these animals had been subjected to radical operative dissections of the mediastinum, with a resection of

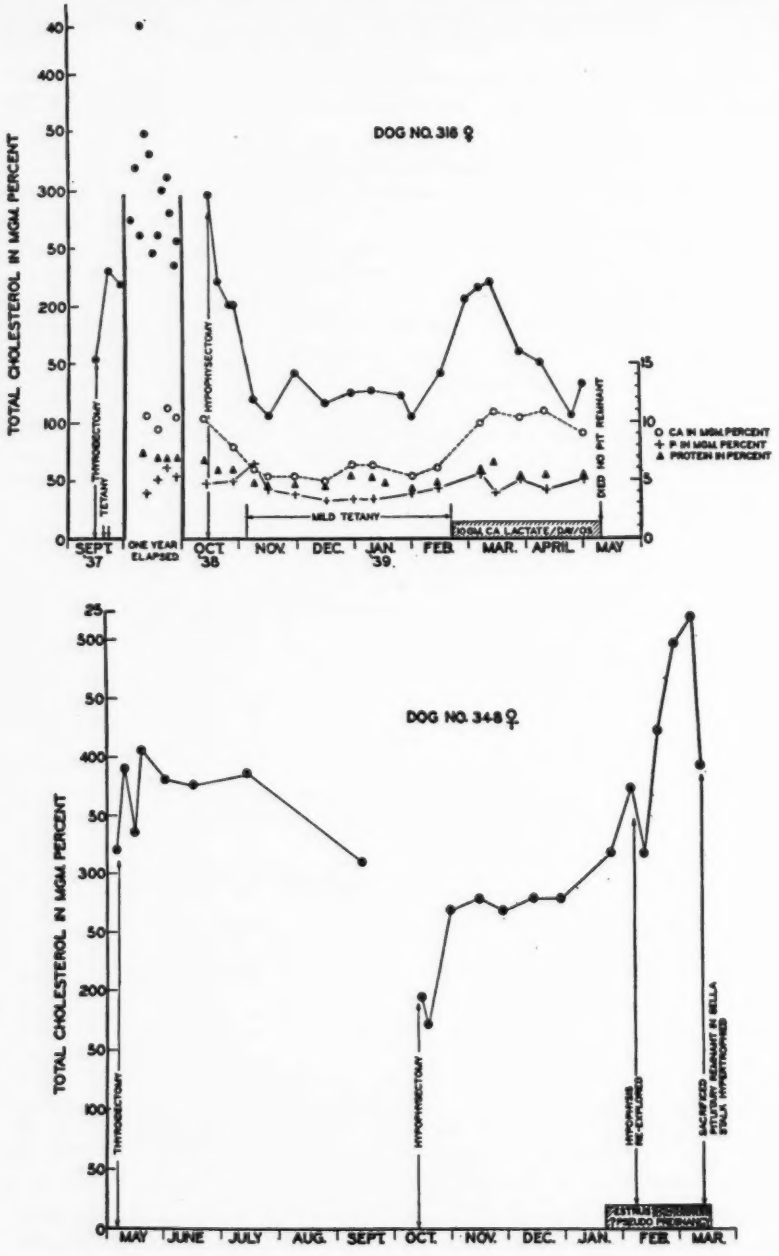

Fig. 1. Effect of thyroidectomy and subsequent hypophysectomy on the plasma cholesterol, serum calcium, phosphorus and proteins of a dog. Fig. 2. The behavior of the plasma cholesterol of a dog in which the hypophysectomy was incomplete.

all visible thymic, fatty, and lymphoid tissue on all sides of the arch of the aorta and in the anterior mediastinum without, however, raising the cholesterol in the plasma.

Hypophysectomy. Six of the thyroidectomized dogs with sustained high levels of cholesterolemia were hypophysectomized (table 1, fig. 1). This operation was followed by a gradual fall in the amount of plasma cholesterol which reached normal values in approximately 4 weeks, and later in several instances fell to remarkably low levels. One of these animals (348 ♀) showed only a transient fall in the cholesterol following

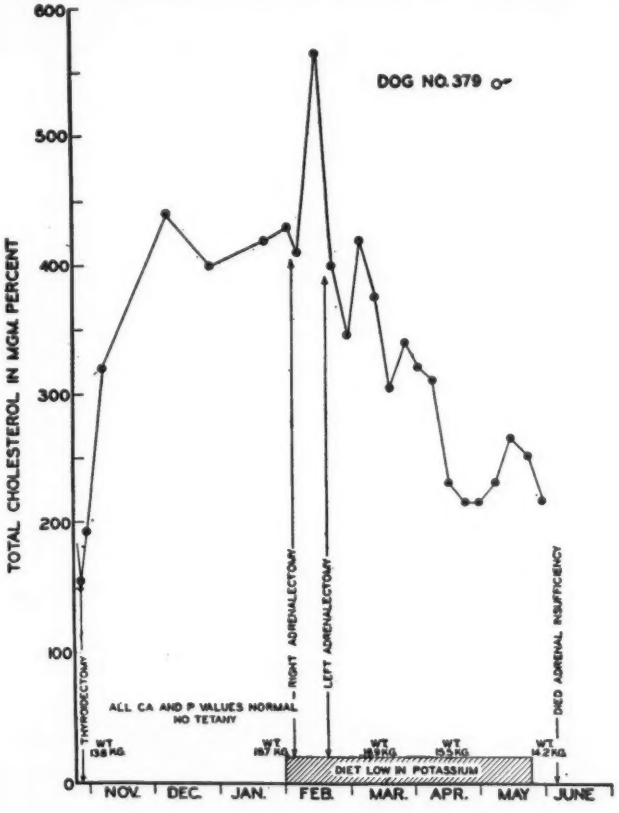

Fig. 3. Effect of thyroidectomy and subsequent adrenalectomy on the plasma cholesterol of a dog.

hypophysectomy, and this dog ultimately had a period of estrus at which time the cholesterolemia was greatly enhanced. Autopsy revealed a remnant of anterior lobe in the sella (fig. 2).

These observations are interpreted to indicate that the hypophysis is actively involved in the production of the hypercholesterolemia of thyroidectomy. This effect may be due to an excessive secretion of one of its hormones which regulates cholesterol or fat metabolism, or it may be the direct and unopposed effect of one of the subsidiary ductless glands which invariably atrophy following hypophysectomy.

Without success on two occasions attempts were made to re-elevate the choles-

terol values of thyroidectomized-hypophysectomized dogs by the daily injection of a crude alkaline extract of bovine anterior lobes. The physical condition of the animals was apparently too precarious to withstand the drastic effects of the injections of crude material, which brought about early fatalities without raising the already low cholesterol values of the animals.

Adrenalectomy. Three of the test animals (thyroidectomized dogs with sustained hypercholesterolemia) were bilaterally adrenalectomized, and without cortical hor-

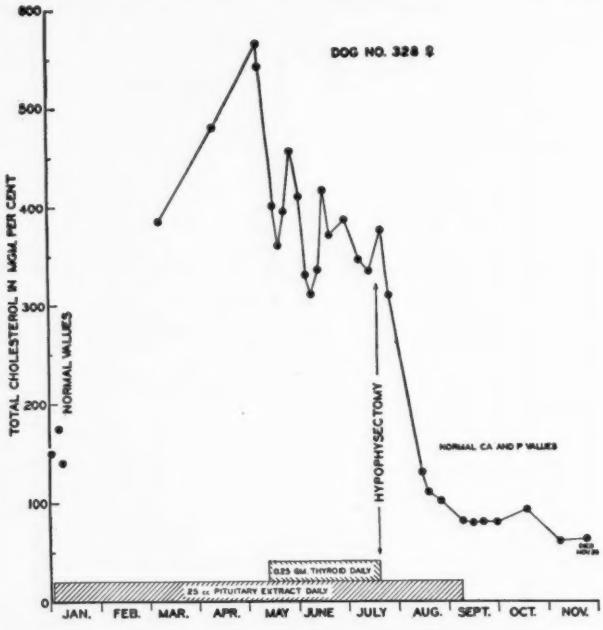

Fig. 4. Effect of anterior pituitary extract and subsequent hypophysectomy on plasma cholesterol.

mone were maintained postoperatively by the feeding of a diet low in potassium and rich in sodium. These procedures, like hypophysectomy, also resulted in a marked reduction of the plasma cholesterol values (fig. 3). In 2 of these animals the fall was as rapid as that produced by hypophysectomy, while in a third the cholesterolemia subsided more slowly and the value did not reach normal in the 2 months' period of observation. At the end of this time the dog was fed a meat diet and adrenal insufficiency was induced. The results in these 3 animals, despite the fact that the cholesterol values fell after adrenalectomy, are difficult to interpret, because of the fact that the clinical condition of the first 2 was so poor. This was possibly due to their poor appetites, which nullified the value of the low potassium diet. The third animal probably represented the true picture, if one may be permitted to hazard a guess.

Additional observations. Three thyroidectomized-hypophysectomized dogs began to have convulsions (fig. 1) approximately 40 days after the hypophysectomy despite adequate carbohydrate therapy. The first animal died without the cause of the convulsions having been discovered. The cholesterol had been at the low level of 60 to 80 mg. % during the 3 weeks' period of convulsions. The second instance of convul-

sions (fig. 1) occurred in a similar animal when the cholesterol had receded to the level of 65 mg. %. At this time the calcium value of the blood serum was found to be 3 mg. per 100 cc. Therapy with calcium immediately stopped the convulsions, and the cholesterol value returned to normal. Tetany later recurred and again was completely relieved by calcium therapy. A third animal showed the same phenomenon, and again calcium therapy appeared to cause some elevation of the cholesterol of the plasma.

On reviewing the history of these dogs our notes mentioned that following their thyroidectomies each had a transient period of tetany despite our care at operation to preserve the parathyroids. In the second and third instances for months before the hypophysectomies were performed normal calcium values of the blood serum were obtained.

An entirely satisfactory explanation for the tetany observed in these animals following hypophysectomy is not apparent. It is conceivable that these observations support the claims of those who have proposed an hypophyseal parathyrotropic hormone (16, 17, 18), which with respect to the parathyroid is presumably analogous to the thyrotropic hormone and the thyroid. On the other hand, the animals might be compared to chronically underfed animals with pronounced malnutrition, despite their adipose appearance associated with hypothyroidism. It does not seem likely that the hypocalcemia was the direct result of restriction of calcium intake after hypophysectomy because in several experiments there was no difference in the quantity of food eaten before and after hypophysectomy. In other words, the thyroidectomized animal had as poor an appetite before the hypophysis was removed as he had afterward. However, with the onset of tetany these animals refused all food and had to be maintained on glucose infusions until they were given calcium chloride intravenously.

The injection of pituitary extract. An attempt was made to determine whether the plasma cholesterol of normal dogs might be elevated by the injection of crude anterior pituitary extract. This was suggested by observations made (by K.W.T.) on a dog (*Alice*) that has had plasma cholesterol values of 400 to 900 mg. % for a period of 5 years during which the animal has received daily injections of a crude sheep pituitary extract. For the present study may be quoted the records of 3 other dogs that developed hypercholesterolemia followng injections of this same extract. Although the injections caused a marked and sustained elevation of the plasma cholesterol, it must be borne in mind that this may have been an indirect effect. Indeed, this apparently was the case as was shown by certain observations on 2 of 3 dogs[3] as follows.

1) A female shepherd dog for a period of 1 year had daily injections of sheep pituitary extract which caused the animal to become obese and to show the clinical signs of deficiency of the thyroid. The plasma cholesterol rose to 313 mg. %. The blood serum contained antihormones which ultimately inactivated her own pituitary gonadotropic and thyrotropic hormones. At autopsy the thyroid gland appeared to be inactive, and the anterior pituitary showed the changes characteristic of thyroidectomy and of castration (19).

2) A female mongrel dog (fig. 4) was injected daily with a similar sheep pituitary extract until the plasma cholesterol value reached a sustained high level. The animal was then fed 0.25 gm. of thyroid daily while the injections were continued. The thyroid feeding apparently caused some recession in the height of the cholesterol value, which, however, became fairly constant at a level considerably higher than normal. The thyroid feeding was then omitted, and the hypophysis was removed while the pituitary injections were continued. The ablation of the pituitary body caused a prompt fall in the plasma cholesterol in precisely the same time and to the same degree that customarily was the case after this operation in thyroidectomized dogs. The continuation of injections of pituitary extract was ineffective for the maintenance of the cholesterolemia after the hypophysis had been removed.

[3] These animals were also the subjects of antihormone experiments reported elsewhere (19).

SUMMARY

The hypercholesterolemia of seven thyroidectomized dogs was abolished by hypophysectomy of the animals. Adrenalectomy produced a similar effect in three thyroidectomized dogs, but the clinical course of two of these animals was such that a final interpretation of these particular results is not possible.

The hypophysectomy was followed in three instances by a striking hypocalcemia with tetany, which occurred at the time the cholesterol values were extremely low. The administration of calcium gave relief from the tetany, and this on one occasion was associated with a temporary rise in the plasma cholesterol values.

Daily injections of an extract of sheep pituitaries caused an elevation of the plasma cholesterol of three dogs. This effect in all probability was due primarily to an inactivation of the animals' own thyroids by antihormones rather than to a direct effect of the extract on tissue cholesterol metabolism.

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REFERENCES

1. Blumgart, H. L., S. A. Levine and D. B. Berlin: *Arch. Int. Med.* 51: 866. 1933.
2. Boyd, E. M., and W. F. Connell: *Quart. J. Med.* 5: 455. 1936.
3. Gildea, E. F., E. B. Man and J. P. Peters: *J. Clin. Investigation* 18: 739. 1939.
4. Hurxthal, L. M.: *Arch. Int. Med.* 53: 762. 1934.
5. Gardner, J. A., and H. Gainsborough: *Brit. M. J.* 2: 935. 1928.
6. Dodds, E. C., and J. D. Robertson: *Lancet* 125: 1137. 1933; 125: 1197. 1933.
7. Hurxthal, L. M.: *Arch. Int. Med.* 53: 825. 1934.
8. Hurxthal, L. M., and H. M. Hunt: *Ann. Int. Med.* 9: 717. 1935.
9. Allers, W. D., and E. C. Kendall: *Am. J. Physiol.* 118: 87. 1937.
10. Sevringhaus, A. E., G. K. Smelser and H. M. Clark: *Proc. Soc. Exper. Biol. & Med.* 31: 1127. 1934.
11. Bloor, W. R.: *J. Biol. Chem.* 24: 227. 1916.
12. Sackett, G. E.: *J. Biol. Chem.* 64: 203. 1925.
13. Tisdall, F. F.: *J. Biol. Chem.* 56: 439. 1923.
14. Kuttner, T. T., and H. R. Cohen: *J. Biol. Chem.* 75: 517. 1927.
15. Man, E. B., and E. F. Gildea: *J. Clin. Investigation* 15: 203. 1936.
16. Anselmino, K. J., F. Hoffman and L. Herold: *Klin Wchnschr.* 12: 1944. 1933; *Ibid.* 15: 45. 1934.
17. Hertz, S., and A. Kranes: *Endocrinology* 18: 350. 1934.
18. Houssay, B. A.: *Harvey Lectures* 31: 116. 1936; *New England J. Med.* 214. 1128. 1936.
19. Sevringhaus, A. E., and K. W. Thompson: *Am. J. Path.* 15: 391. 1939.

ELECTROPHORESIS OF ANTIHORMONE SERA[1]

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ANTIHORMONES in animal sera are believed to be immune bodies of an unusual variety (1). This conclusion is based upon indirect evidence, chiefly their physiological actions, about which more is known than their chemical and physical characteristics. These substances appear to be contained in the pseudoglobulin fraction of sera, are thermolabile, and are precipitated from serum by 80% acetone or by half-saturation with sodium sulfate (1). Further observations on the physicochemical nature of the antihormones is offered in this communication, which reports the results of an electrophoretic study of gonadotropic antisera.

To obtain the electrophoretic diagrams the sera were examined in a modified Tiselius apparatus which was equipped with the schlieren scanning device of Longsworth (2). In the present apparatus a slit 1 mm. in width was used (3, 4) in place of a single edge which in the scanning device moves vertically while the film at the same time moves horizontally. The descending boundaries (protein into protein) were scanned for the reasons advanced by Longsworth and MacInnes (5); no beta-disturbances (6) were observed as may be seen in the photographs. The area under each peak is a function of refraction of the light by a protein constituent, and is consequently proportional to the concentration of that protein component in the serum.

The separation experiments were carried out in a macro-Tiselius apparatus which has a capacity of 330 cc. of fluid (4, 7). Each individual compartment contains 35 cc. The PH of the dialyzed solutions was 8.0; the supernatant fluid was 0.017 M phosphate buffer. The sera studied were obtained from 3 goats, each of which was injected subcutaneously daily with a gonadotropic extract prepared by saline extraction of an acetone-dried powder of whole human pituitary glands. The daily dose was the soluble portion of 75 mg. of powder. At certain intervals the electrophoretic pattern and the antihormone activity of these sera were examined. The inhibitory power of the serum was estimated by the ability of the serum to inactivate, in test rats, the gonadotropic action of an extract containing the soluble portion of 10 mg. of human pituitary power. The data for *goat 1* are presented in figure 1 and table 1, and they illustrate that the concentration of the gamma-globulin of the serum increases with the increasing antihormone activity of the serum. Similar results were obtained with the sera of *goats 2* and 3.[3]

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[2] Finney-Howell Research Foundation Fellow, 1939–41.

[3] At first glance one might assume that the augmentary power (designated "A" in table 1) was in the beta-globulin fraction. The serum of *goat 2*, however, contained 9 to 11% of the total serum protein in the beta-globulin fraction even before the animal was injected with the pituitary extract. This fraction persisted in the same relative concentration after the 60th day of injection; it was not found after the 100th day of injection. The gamma-globulin concentration, however, in *goats 2* and 3 like that of *goat 1*, was greatly increased when the animals received daily injections of the pituitary extract.

TABLE 1. THE CHANGES IN THE ELECTROPHORETIC COMPONENTS OF ANTISERA OF *goat 1* IN RELATION TO THE ANTIHORMONE ACTIVITY OF THE SERUM AND THE DURATION OF THE INJECTION PERIOD. SEE FIGURE 1 FOR THE DIAGRAMS OF THESE SERA

Day of injections	Inhibitory power[1]	% Total protein				A/G Ratio, Tiselius method
		Albumin	Globulin α	Globulin β[3]	Globulin γ	
0	0	62.9	9.6	□	27.5	1.7
0	0	66.1	6.4	0	27.5	1.9
15	A	55.0	5.7	12.0	27.3	1.2
15	A	49.8	3.9	18.7	27.6	.99
30	1	55.0	6.1	5.5	32.8	1.2
30	1	56.1	6.3	6.1	31.5	1.3
45	2	51.3	5.1	□	43.6	1.1
45	2	50.5	7.8	0	41.7	1.0
75	3	48.9	7.8	0	43.3	.96
75 Partially Purified[2]	4	16.5	12.3	0	71.2	—
160	2	40.4	6.7	0	52.9	.68
160 Partially Purified[2]	4	4.7	7.2	□	88.1	—

[1] The symbols expressing inhibitory action upon the test dose of human pituitary gonadotropic extract have the following meaning:
0—no action whatever
A—slight augmentary effect
1—inhibited in doses greater than 1.5 cc.
2—inhibited in doses greater than 0.5 cc.
3—inhibited in doses greater than 0.25 cc.
4—inhibited in doses greater than 0.10 cc.

[2] Partially purified by precipitation of globulins with Na_2SO_4.

[3] If a small amount of β globulin were present, its resolution from the large amount of γ globulin would be difficult, and may not have been detected.

The components of an active serum (from *goat 3*) were separated in the macro-Tiselius apparatus in order to determine whether the observed increase in gamma-globulin might be more definitely correlated with the formation and accumulation of

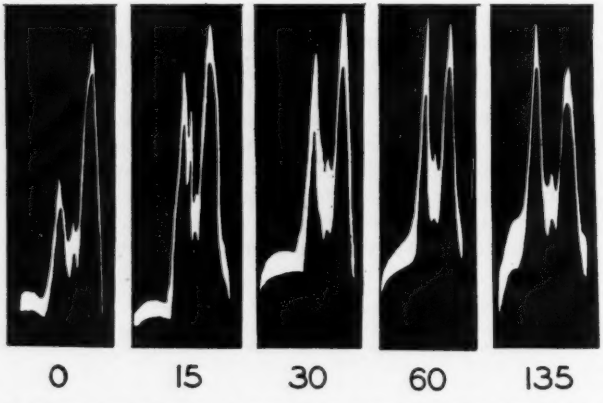

Fig. 1. ELECTROPHORETIC BEHAVIOR OF SERUM OF *goat 1* THAT RECEIVED DAILY INJECTIONS OF HUMAN PITUITARY EXTRACT. Descending boundaries (cathodal limb); albumin on right, gamma-globulin on left, numbers indicate days.

TABLE 2. THE MINIMUM EFFECTIVE DOSE OF INDIVIDUAL FRACTIONS OF THE SEPARATED SERUM IN TERMS OF TOTAL PROTEIN, AND OF EACH OF THE PROTEIN COMPONENTS

Fractions of serum	Protein concentration	Min. effective dose of antihormone		% Total protein		
		Fraction	Protein	Albumin	Globulin Alpha	Globulin Gamma
	mg./cc.	cc.	mg.			
C_3	4.90	0.25	1.3	0	0	100
C_4	10.60	0.25	2.7	27.2	12.1	60.7
M	13.60	0.25	3.4	47.2	9.3	43.5
A_4	9.50	2.0	19.0	74.9	13.7	11.4
A_3	4.62	In 5 cc. no activity	No activity in 23.0	100	0	0
Undiluted original serum	74.90	0.05	3.7	47.2 44.8[1]	52.8 55.2[1]	

[1] Chemical method with salt precipitation.

the antihormones. The separated contents of certain chambers, namely the two intermediate anodal, the two intermediate cathodal, and the three combined middle sections were each analyzed for antihormone activity, for protein nitrogen by a micro-Kjeldahl procedure, and for electro-chemical homogeneity in the analytical Tiselius apparatus.

The data (table 2, fig. 2) show that the entire antihormone activity resided in the gamma-globulin fraction, and that the antihormone activity of the contents of a particular chamber was proportional to the amount of gamma-globulin present in that chamber. The results obtained illustrate the applicability of the macro-Tiselius technic for the preparation of homogeneous proteins: in a single experiment two electro-

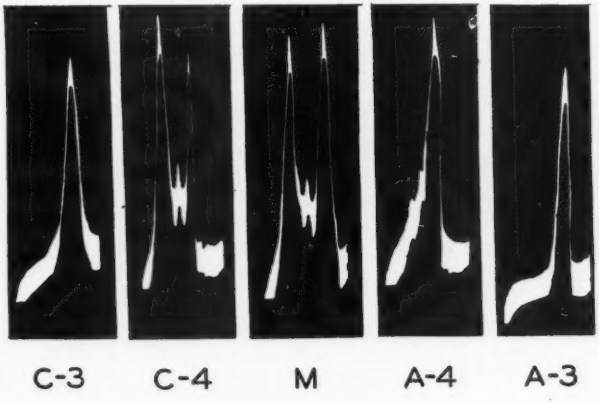

Fig. 2. SEPARATION OF AN ACTIVE ANTIHORMONE SERUM FROM *goat* 3 IN THE MACRO-TISELIUS ELECTROPHORETIC APPARATUS. The fraction nearest the anode is labelled *A-3*, that nearest the cathode, *C-3*. *M* is the middle fraction, and *A-4* and *C-4* are intermediate fractions. The schlieren photographs were obtained by subjecting the contents of the chambers of the separation apparatus to electrophoresis in the analytical cell (descending boundaries; albumin on right, gamma-globulin on left).

chemically homogeneous fractions each of 35 cc. (containing about one-half per cent protein) were obtained from a mixture of three proteins; one fraction represented gamma-globulin and the other albumin. Two intermediate fractions greatly enriched in gamma-globulin and albumin respectively were obtained in similar quantities. Although the "normal" gamma-globulin and the antihormones appear to have identical electrochemical properties, this does not preclude the possibility that they might yet be separated by other methods.

The writers gratefully acknowledge the aid of Dr. Kurt G. Stern, who originally collaborated in this work, and whose advice was generously given throughout the experiments.

REFERENCES

1. THOMPSON, K. W.: *Physiol. Rev.* In press.
2. LONGSWORTH, L. G.: *J. Am. Chem. Soc.* 61: 529. 1939.
3. FELL, N., K. G. STERN AND R. D. COGHILL: *J. Immunol.* 39: 223. 1940.
4. STERN, K. G., AND D. DUBOIS: Unpublished work.
5. LONGSWORTH, L. G., AND D. A. MACINNES: *J. Am. Chem. Soc.* 62: 705. 1940.
6. LONGSWORTH, L. G., T. SHEDLOVSKY AND D. A. MACINNES: *J. Exper. Med.* 70: 399. 1939.
7. TISELIUS, A.: *Bull. New York Acad. Med.* 16: 751. 1940.

SPONTANEOUS INTERSEXUALITY IN THE RAT

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BECAUSE of the reports on the feminizing influence of estrin on rat fetuses, the repeated, spontaneous occurrence of a similar, but more pronounced type of intersexuality in our rat colony seems worthy of brief description, especially as no similar case appears to have been recorded. The abnormality was first noted in the fall of 1938, and during the ensuing two years, eight cases, in litters of 3, 2 and 3,

Fig. 1. VAGINA AND TESTES OF INTERSEX RAT AT AGE OF ABOUT NINE MONTHS. Fig. 2. PELVIC ORGANS OF SAME RAT. URINARY BLADDER AND TESTES ARE VISIBLE. NOTE ABSENCE OF SEMINAL VESICLES AND PROSTATE. Fig. 3. PELVIC ORGANS OF NORMAL RAT, FOR COMPARISON WITH FIGURE 2.

have been observed. All were exactly alike and may be described together; two were observed over periods of six months and one year.

Externally, these rats were females in every respect. The vaginae were blind pouches, about one-half centimeter deep, and opened at the age of 3 months. Nipples indistinguishable from those of female rats were present, which is significant, as the male rat does not possess even rudimentary nipples. Internally, no trace of ovaries, uteri or Fallopian tubes could be found. Each animal possessed a pair of small, cryptorchid testes, but no vestige of accessory male sex organs whatever. The testes were attached to ligaments, but no tubular structure connected them with the exterior (fig. 1–3).

Histologic studies were made of the hypophysis, adrenals and testes. Nothing abnormal could be detected in the first two organs. The testes were compared with those of normal males and with testes from rats rendered cryptorchid for the same

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periods of time. A very definite difference was noted, in that the interstitial tissue surrounding the tubules was condensed into a dense mass, forming a sort of collar around each tubule. This collar did not merge gradually into the remaining interstitial tissue but appeared to be part of the tubule itself. There is, of course, no spermatogenesis, as the testes were cryptorchid (fig. 4, 5). In addition, the animal kept alive for one year had several peculiar, sharply defined bodies resembling corpora lutea in each testis (fig. 6). The sections have been examined by several pathologists, but without definite agreement as to their nature. To us they appear to be corpora, although in some respects (as pointed out by one prominent pathologist), they bear some resemblance to adrenal cortex tissue.

The animals kept under observation developed as females in respect to weight gain. At autopsy their adrenals weighed nearly twice as much as those of males of the

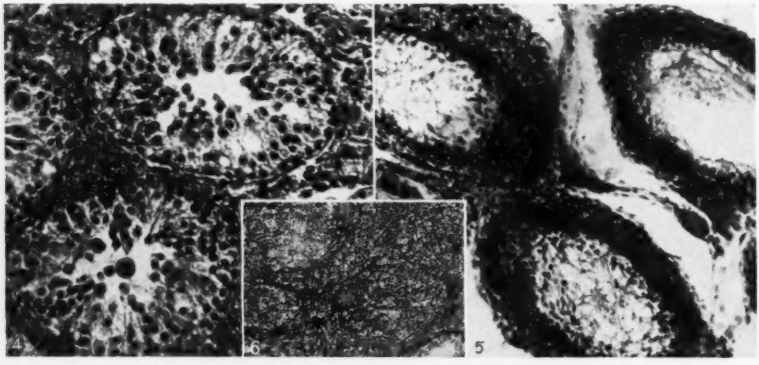

Fig. 4. Testes of rat cryptorchid for six months. For comparison with figure 5. Fig. 5. Section of testes of intersex rat. Note condensation of interstitial tissue around tubules. Fig. 6. Structure resembling corpus luteum. Seminiferous tubules at edge.

same age. It is, of course, well known that the adrenals in the female rat are distinctly heavier than those of the male. Vaginal smears were taken daily over long periods, a small number of leucocytes and a few large squamous cells were present, but there was no cyclic change. Sexually, they were apparently considered to be females by males placed in their cage, they themselves betrayed no interest in either sex.

DISCUSSION

In this laboratory hundreds of rats are used every week and it is therefore impossible to keep genealogic records. We have attempted to locate parents by isolated breeding, but have not succeeded. Therefore nothing is known of the genealogy of this trait.

Greene, Burrill and Ivy,[1] have recently reported the production of feminized male fetuses by the injection of pregnant rats with large amounts of estrogenic substance. Although we have been engaged in several unrelated studies which involved estrin administration, we feel certain that no error could have occurred which would explain the appearance of these inter-sexes on the basis of estrin administration. Nothing like the amounts of estrin found by the above workers to be necessary have ever been injected into rats of our colony; in addition, their rats did not, as a rule, deliver spontaneously, Caesarian section being necessary. We therefore feel that these animals represent true cases of spontaneous intersexuality.

[1] Greene, R. R., M. W. Burrill and A. C. Ivy: *Science* 88: 130. 1938.

A SPONTANEOUS ABNORMALITY OF THE RAT'S OVARY ACCOMPANIED BY MASCULINIZATION OF THE GENITALIA[1,2]

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TUMORS of the glands of internal secretion have been described frequently by clinicians and experimentalists. That such glandular neoplasms may secrete abnormal kinds and amounts of hormones and directly or indirectly disturb the endocrine balance in the organism has been demonstrated in many instances. From studies on spontaneous glandular abnormalities it may become possible, after the experimental simulation of the defect, to ascribe specific functions to particular tissues and cells comprising the organ.

Studies upon the secretory capacity of the ovary subsequent to the onset of various spontaneous and experimental lesions have been of some value in interpreting the normal gonad. It has not been possible, however, to determine conclusively the exact tissues which are responsible for the synthesis of the several ovarian secretions.

The present paper deals with a spontaneous ovarian defect which seems to be associated with the impaired secretion of gonadotropic hormones by the hypophysis cerebri. Evidence is submitted indicating that these ovaries secrete estrogenic hormones for prolonged periods and that they also exert an androgenic action.

MATERIAL AND METHODS

Among a strain of inbred rats procured from a local pet shop, occasional females were found which did not litter, although caged indefinitely with fertile males. All of these animals possessed an abnormality of the external genitalia which made their detection possible without resorting to exploratory laparotomy. In some of the animals, large palpable nodules of a lobulated nature could be felt in the ovarian region. From approximately one thousand animals, 7 pathological individuals were found over a period of 3 years. All of these were present in different litters.

Estrous cycles were followed by means of vaginal smears in order to determine the periodicity of the hypophysis and ovary. The androgenicity of the gonads was established by transplanting seminal vesicles and ventral prostatic lobes into the anterior chamber of the eyes of these females, and by transplanting the abnormal ovaries beneath the renal capsule of bilaterally orchotomized males. At autopsy, the gonads, sex accessories, adrenals, thyroids and hypophyses were preserved for histological examination. An attempt was made to simulate this abnormality by the administration of varying amounts of crystalline sex hormones to normal females.

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[2] The author gratefully acknowledges the technical assistance of Mr. W. L. Burkhardt and Miss Rita Haffen.

OBSERVATIONS AND EXPERIMENTS

A. *Protocols*

Rat 1. This animal was placed in a breeding cage at approximately 100 days of age. An abnormality of the external genitalia was detected when she was 155 days of age. Vaginal introitus had occurred but a narrow septum extended across the interior of the vaginal orifice. This rat was autopsied when 309 days of age. By this time the vaginal septum had not been perforated though she had been caged with fertile males. Vaginal smears were recorded for 57 days previous to autopsy. Copulations were not observed even when the vaginal smear was of the estrous type. When the animal was autopsied large pus-filled cysts were observed bilaterally in the regions of the fallopian tubes. The cranial ends of the uteri were twisted and contained a thick caseous material. The ovaries were small, possessed vesicular follicles, and contained no macroscopically visible corpora lutea. The interfollicular regions of both ovaries contained masses of whitish tissue, whereas in the region of the hilus light brownish patches were observed.

Rat 4. This was one of the first abnormal individuals encountered. She had been kept for 210 days with males and attracted attention because of her inability to become impregnated and to deliver litters. Vaginal smears were recorded for a period of 2 months, the animal then being autopsied at the age of 270 days. Pyosalpingitis and pyometria occurred on the left side only. The left bursa was inflamed and distended with thick yellowish material. In several places the bursa adhered to the surface of the ovary. The right and left ovaries appeared identical when studied under the dissecting microscope. No corpora lutea were present. The areas between the vesicular follicles were occupied by clumps of whitish tissue.

Rat 618. The abnormality of this individual was detected and recorded when she was 131 days of age. Vaginal smears were taken for a period of 60 days. At 191 days of age this animal was laparotomized and the ovaries removed for transplantation into the kidneys of an adult male castrate. The ovaries appeared as in the preceding animals. At laparotomy no evidence of pyosalpingitis or pyometria was detected on either side. A portion of the right uterus was excised and preserved for histological purposes. The animal was maintained in a bilaterally oöphorectomized state for a period of 124 days and finally autopsied at 315 days of age. At autopsy, the uterus was found to be quite small and approximated the castrate condition. The vagina was also of the castrate type. It was felt that as the result of oöphorectomy there had been some decrease in the size of the glans clitoridis and atrophy of the erectile tissues of the clitoris. The cleft condition of the phallus persisted.

Rat 933. At the age of 93 days a seminal vesicle from a 10-day-old donor was inserted into the anterior chamber of each eye. After 30 days, these eye grafts underwent visible enlargement and became distended with secretion. The right eye graft was removed after it had persisted for a period of 66 days. Histological examination revealed that it had been maintained in a secretory condition. Vaginal smears were taken and recorded for 57 days previous to laparotomy at the age of 159 days. This animal never copulated successfully since the thin vaginal septum remained unbroken. At laparotomy the two ovaries were removed and transplanted to the kidneys of a male castrate. Both uteri and a portion of the vagina were removed and fixed for histological examination. There was no inflammation of either uterus or fallopian tube. Autopsy was performed 41 days later. After castration of the host, the seminal vesicle transplanted to the left eye decreased in size and no longer appeared to be distended by secretion. Histologically there was no evidence of secretion in this graft although viable seminal vesicle tissue was still present.

Rat 886. At the age of 174 days, this animal received ventral prostatic lobes from a 10-day-old donor, these being transplanted to the anterior chambers of the eyes. Autopsy was performed when the animal was 234 days of age. Macroscopically, both eye grafts appeared fibrotic. No evidence of secretion was observed in either graft after fixation and microscopic study. The two ovaries were examined under the dissecting microscope and were found to appear as in the previous pathological animals. One of these gonads was transplanted to the kidney of a castrate male host. The autopsy protocol for this animal states that both right and left ovarian regions were distended tremendously by pus-filled cysts. Vaginal smears were not recorded for this animal.

Rat OT 21. Defective external genitalia were detected in this animal at the age of 38 days. At this age no abnormal growths could be palpated in the ovarian regions. Ventral prostatic tissue from a 9-day-old donor was transplanted to each eye and permitted to persist for 73 days. One of the sectioned grafts contained light areas in the epithelial cells lining some of the acini. Mild inflammation of both tubes and both uteri was detected at autopsy. Vaginal smears were not made.

Rat OT 551. This animal was autopsied at 210 days of age. Successful copulations never occurred since the vaginal septum remained intact throughout life. Both fallopian tubes were cystic. The cranial ends of both uteri were twisted and inflamed. A large amount of purulent material was expressed from both tubes and uteri. In several places the ovaries adhered to the bursae. Each ovary contained vesicular follicles, no corpora lutea, and white masses of tissue between the follicles. Vaginal smears were not recorded.

B. *Vaginal Smears*

Vaginal smears were taken daily from 4 of the animals possessing the spontaneous abnormality of the ovaries, and from one normal litter mate (fig. 1). These observations extended over approximately two months for each individual. *Rat* 935 was a normal litter mate of *rat* 933. The normal animal ran a regular estrous cycle, coming into estrus at intervals of from 4 to 7 days. The vaginal smear histories of defective animals 933, 4, 1, and 618 are strikingly atypical as compared with the normal (fig. 1). A loss of normal ovarian periodicity is evident in all of the diseased individuals. They came into vaginal estrus infrequently but tended to remain in this state for a prolonged period.

Animal 933 (fig. 1) came into estrus only once over a period of 2 months but remained in estrus for 10 consecutive days. For 35 days only diestrous smears were

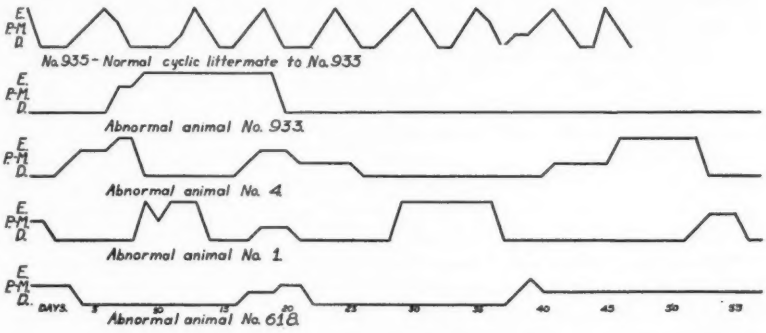

Fig. 1. VAGINAL SMEAR HISTORIES OF 4 PATHOLOGICAL ANIMALS AND ONE NORMAL LITTER MATE.

obtained. Infertile copulations could not have resulted in pseudopregnancy since the vaginal septum was never perforated. During diestrus, a few nucleated epithelial cells and much mucus were obtained in the smears.

Rats 1 and 4 displayed vaginal estrus several times and remained in this state for relatively long periods. During diestrus, a few nucleated and keratinized cells could be found. Mucus was present in the diestrus smears. It is probable that the bacterial inflammation of the tubes and uteri contributed to the prevalence of leucocytes in the smears.

Animal 618 never gave a typical estrous smear. Proestrous smears were obtained for prolonged periods. At all times, mucus was present in the vaginal lumen.

C. *Mating and Fertility*

None of the 7 animals littered although caged with fertile males for most of their lives. All of them were observed for extended periods but copulations were never witnessed even when the females gave the estrous smear. Three of the animals (*rats* 1, 933, and *OT* 551) definitely did not copulate since the thin septum across the vaginal orifice remained intact throughout life.

D. *Defective External Genitalia*

In the 7 animals possessing this type of ovarian neoplasm, the external genitalia were abnormal (fig. 2, 3). This genital defect is characterized by a cleft condition of

the phallus, hypertrophy of the glans clitoridis, and a mild hypertrophy of the erectile tissues of the clitoris. Because of the cleft phallus, the distal portion of the clitorine urethra is not extended beyond the glans clitoridis by means of the preputial folds. The urethral meatus consequently is located at the base of the glans clitoridis. The vaginae were canalized but, in 3 of the animals, a narrow septum extended from the interior of the vaginal orifice toward the urethral meatus. An abnormality similar to this has been produced experimentally by the administration of estrogens during the terminal stages of gestation or during the first few days of postnatal life, 1 to 4.

E. Histology of the Genital Tracts

Ovaries. Both ovaries were removed from 5 of the animals at laparotomy or autopsy and serially sectioned for histological studies. A striking degree of structural uniformity was observed throughout. Corpora lutea were absent. All of the ovaries contained from 5 to 14 large vesicular follicles some of which possessed relatively normal ova. It appears that such follicles could not ovulate on account of the thickened thecal layers. With the exception of certain static primary follicles, signs of atresia were evident in follicles of all stages (fig. 4).

Two distinct types of follicular atresia are recognizable. The first is characteristic of unilaminar and early multilaminar secondary follicles. The nucleus of the ovum becomes pycnotic and the cytoplasm eventually vacuolates and fragments. Phagocytic cells may invade the follicle and remove remnants of the ovum, whereas the granulosa and thecal layers of the follicle persist for a longer time in an apparently normal condition. These anovular follicles consist of one or two layers of cuboidal or columnar granulosa cells surrounded by a normal theca folliculi. They lie a short distance below the surface epithelium, near the periphery of the ovary, and are suggestive of sections of seminiferous tubules from a cryptorchid testis. These structures are definitely anovular follicles rather than modified rete tubules or any medullary derivative which could be regarded as visible evidence of masculinization of the gonad.

The second type of follicular retrogression occurs in late multilaminar secondary follicles and in vesicular follicles. The first visible sign of this type of degeneration is hypertrophy of the cells of the theca interna. The granulosa then becomes disorganized, the first departure from normal appearance being cellular shrinkage and nuclear pycnosis. The products of cellular degeneration accumulate in the inner layer of the granulosa and numerous phagocytes move into these regions. The ovum remains relatively normal until all or most of the granulosa has disappeared. Finally, the atretic follicle collapses leaving a deposit of hypertrophied cells of the theca interna encapsulating a small amount of colloid-like substance which stains deeply with eosin.

The most distinguishing feature of these abnormal ovaries is the deposition and persistence of hypertrophied thecal cells resulting from the second type of follicular atresia. The interfollicular areas of the ovary thus become filled with cords and masses of such epithelioid cells (fig. 4, 5, 6). In these ovaries, the cells of the germinal epithelium vary from squamous to cuboidal and columnar and the possibility that cords of epithelioid cells may push in from the surface cannot be excluded definitely. One can be certain, however, that the majority of the masses of epithelioid cells represent the remnants of degenerated ovarian follicles.

Periovarial bursae. In 4 of the animals the bursae were highly vascularized and fibrotic. Certain regions contain many invading cells of vascular and connective tissue origins. Adhesions between the periovarial membrane and the surface of the ovary were encountered frequently. The bursae were distended with a thick yellowish fluid in instances where the tubes were inflamed severely. The pathological condition of he ovaries cannot be accounted for on the basis of bacterial infection of the bursae or

pressure effects resulting therefrom. In animal 4, only the left bursa was inflamed and the ovary did not differ histologically from the uninflamed ovary on the right. Animals 618 and 933 did not suffer from pyosalpingitis and pyometria but their ovaries were as pathological as the others.

Uteri. Pyometria was present bilaterally in 4 of the animals, unilaterally in one, and absent in 2. In instances where pyometria was present, the cranial ends of the uteri were most severely inflamed. Sections through the cranial portions of such uteri indicated that the myometrium and submucosa were fibrotic and contained many polymorphonuclear leucocytes. In many places the epithelium had undergone squamous metaplasia and the lumen was distended by leucocytes and detritus. The caudal

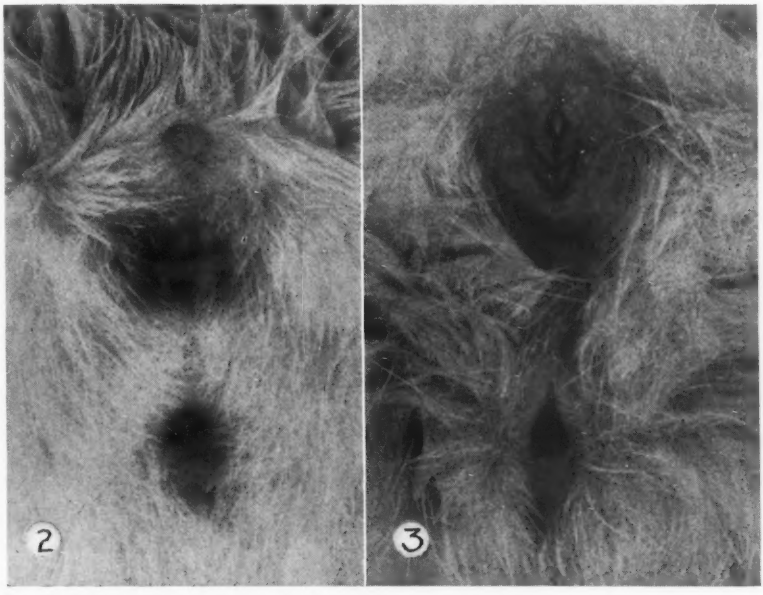

Fig. 2. The external genitalia of a normal adult female rat. Fig. 3. The external genitalia of one of the females possessing the spontaneous ovarian defect. Note the position of the vagina, the cleft phallus, and the hypertrophied glans clitoridis.

ends of these uteri presented a more normal appearace. In most regions the epithelium had undergone squamous metaplasia and well-defined glands were absent. Cystic glandular hyperplasia was evident at certain levels. The stroma was dense and hyalinized throughout but, in the middle and caudal regions, the myometrium approximated normalness. Epithelial metaplasia was mild in animal *OT 21* and cystic distention of the basal ends of the uterine glands was pronounced.

Pyometria appeared in only the left uterus of animal 4. The right uterus, however, had undergone mild squamous metaplasia and cystic glandular hyperplasia was evident at certain levels. The stroma of both uteri was dense and somewhat hyalinized.

Pyosalpingitis and pyometria did not appear in animals 618 and 933. Examination of a mid-segment of the right uterus, removed at laparotomy, revealed extensive epithelial metaplasia and fibrosis of the stroma. After bilateral oöphorectomy for a period of 124 days, the left uterus regressed and assumed the castrate condition. Squamous

metaplasia, cystic distention of the glands, and hyalinization of the stroma were found in both uteri of rat 933.

Vaginae. Some degree of vaginal mucification was observed in all of the intact animals. This explained the presence of mucous secretion so uniformly present in the smears. The degree of mucification never approximated that characteristic of the terminal stages of normal pregnancy but did approximate that of the pseudopregnant

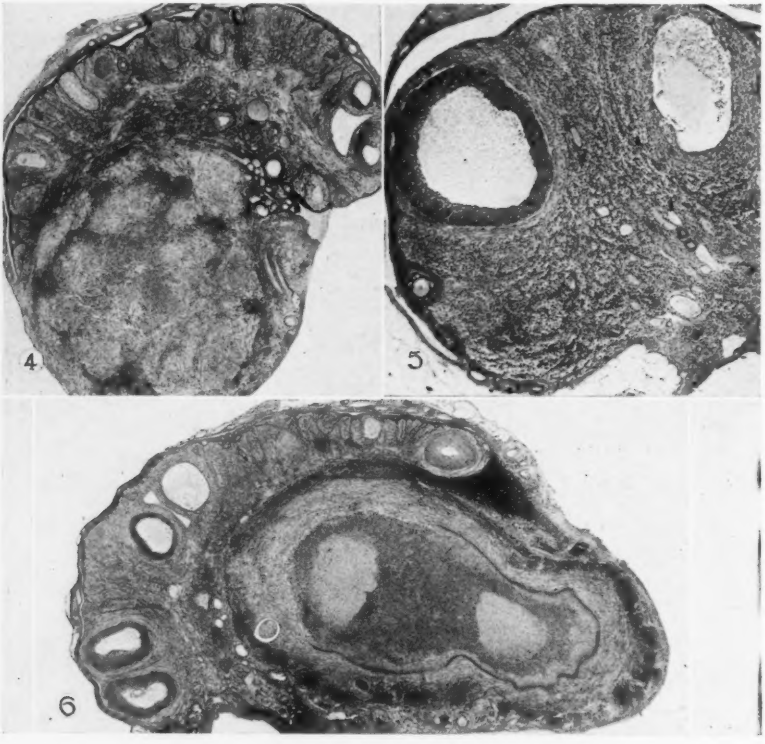

Fig. 4. SECTION OF AN OVARY FROM ONE OF THE ABNORMAL ANIMALS. Observe huge cyst of the fallopian tube below the ovary, and the numerous atretic follicles. Fig. 5. A HIGHER MAGNIFICATION OF A PORTION OF ONE OF THE PATHOLOGICAL OVARIES. Note thickening of the theca interna in the 2 vesicular follicles and the persistence of masses of thecal cells in the stroma. Fig. 6. SECTION OF AN ABNORMAL OVARY AND A PUS-FILLED CYST OF THE FALLOPIAN TUBE. Observe vesicular follicles, various stages of atresia, and persistence of hypertrophied thecal cells.

rat. The vaginal epithelium of some of the animals was thickened very appreciably and a thick layer of cornified cells intervened between this and the superficial layer of mucified cells. The mucified layer was absent at certain levels and the cornified cells were loosened and freed into the vaginal lumen.

The muscle layers and lamina propria of the vagina were fairly normal. The only abnormality of the lamina was in two of the animals where pyometria was severe and extensive. In these cases aggregations of lymphoid cells were observed infrequently just below the vaginal epithelium.

Subsequent to the bilateral removal of the ovaries and tubes, the vagina reverts

to the castrate condition. The epithelium becomes thin and many leucocytes appear in it and in the lamina propria.

F. Transplantation Experiments

Since the abnormal females appeared infrequently and unexpectedly in the litters, some of the most pertinent experiments were not performed. Nevertheless, the limited attempts to test the androgenicity of the ovaries are reported herewith.

At 191 days of age, *rat 618* was laparotomized and both ovaries were transplanted below the kidney capsules of an adult male which had been castrated previously for a period of 67 days. Both grafts incorporated and were not removed until the male host was autopsied 137 days after insertion of the ovarian transplants. The sectioned grafts contained cystic follicles and no normal corpora lutea. Masses of hypertrophied and partially luteinized thecal cells were conspicuous throughout the grafts. The seminal vesicles of the host were enlarged but there was no histological evidence of secretion. The enlargement of the glands was due to hypertrophy of the smooth musculature. The ventral prostatic lobes were larger than in long-time castrates. Sections of the ventral prostate indicated that some of the acini were lined by high columnar cells in which light areas were distinctly visible. Most of the acini were lined by low cuboidal or squamous cells and light areas were not present. It is evident from this experiment that there was sufficient androgen produced to induce and maintain some degree of secretion in the ventral prostate but not enough to cause secretion in the seminal vesicles.

When *rat* 933 was 93 days of age seminal vesicles from a 10-day-old donor were transplanted into the anterior chamber of each eye. After 30 days both grafts enlarged appreciably and became distended by secretion. The right graft was removed and fixed after persistence for 66 days. Microscopical studies indicated that the epithelial cells were columnar and that they contained normal secretory granules. The musculature was hypertrophied. Secretion filled and distended the acini. The host was bilaterally oöphorectomized at the age of 159 days and the graft in the left eye was removed and fixed 41 days later. The left graft had regressed and no evidence of secretion was discernible microsocpically. Thus the intact ovaries of this type are capable of inducing and maintaining secretion in incorporated grafts of the seminal vesicles.

At autopsy the two pathological ovaries from this female (933) were dissected out and transplanted to the kidneys of a castrate male. Neither ovarian graft was recoverable after 61 days and the male accessories were of the castrate type.

Ventral prostatic lobes from a 10-day-old donor were transplanted to the anterior chambers of abnormal female *886* when the latter was 174 days of age. Both eye grafts persisted as small fibrotic nodules and there was no evidence of secretion. One of the ovaries removed from the host at autopsy was transplanted to the kidney of a castrate male. Large cystic follicles were present in this ovarian graft when removed 84 days after insertion. Secretion in the male accessories was not induced. The seminal vesicles were enlarged as the result of smooth muscle hypertrophy. This single ovarian graft apparently liberated estrogen but not enough androgen, if any, to maintain secretion in the male accessory glands.

Ventral prostates from a 9-day-old donor were transplanted to the anterior eye chambers of female *rat OT 21* when she was 38 days of age. The grafts were permitted to persist for 73 days. One prostatic graft was fibrotic whereas the other contained acini in which light areas were visible.

DISCUSSION

The observations and experiments herewith reported indicate that the ovaries of

these abnormal animals are both hyperestrogenic and mildly androgenic. The estrogenicity of these gonads is indicated by the squamous metaplasia and cystic glandular hyperplasia of the uteri, by the cornification of the vaginal epithelium, and by the hypertrophy of the musculature of the seminal vesicles when transplanted to the eyes of these animals. Ovarian androgenicity is attested by the fact that secretion is induced and maintained in some of the seminal vesicles and ventral prostates transplanted to the eyes of the pathological females, and by the fact that transplanted ovaries of this type occasionally maintain secretion in the ventral prostatic lobes of castrate male hosts. The enlargement of the glans clitoridis and the erectile tissues of the clitoris in these intact females is presumptive evidence of an androgenic effect. The regression of the transplanted seminal vesicle subsequent to bilateral oöphorectomy is proof that the ovary rather than an extra-gonadal site is responsible for the production of the androgenic substance.

Androgenicity of the mammalian ovary. That the ovary may become capable during unusual or abnormal circumstances of liberating an androgenic substance is not a new concept. Steinach and Kun (5) reported that the luteinized ovaries of the guinea pig might exert a masculinizing effect. Lipschütz (6) found that an ovarian graft persisting for 3 years in a castrate male guinea pig had restored fully the seminal vesicles and prostatic glands of the host. The excellent experiments of Hill and collaborators (7–11) indicate that ovarian homotransplants into the ears of castrate mice frequently become capable of maintaining secretion in both the seminal vesicles and prostates. A similar androgenic action of ovarian grafts persisting in the ears of rats has been reported by Deanesly (12, 13). She did not find that the androgenicity of the ovarian grafts was conditioned by temperature as Hill maintains for mice.

Several workers have shown that crystalline progesterone produces androgenic effects (14, 15, 16). According to Greene and collaborators relatively large amounts of progesterone are required in order to maintain the prostates and seminal vesicles of castrate males, the hormone being much more effective when administered subcutaneously than when given intraperitoneally. It has not been proved that the chemical configuration of the progesterone molecule is not altered by the organism before masculinizing effects are elicited. Nelson (17) found that progesterone resembled androgens inasmuch as it was capable of maintaining spermatogenesis in the gonads of hypophysectomized rats. Deanesly and Parkes (18) observed that testosterone acetate and propionate produced vaginal mucification in oöphorectomized immature rats, an effect once attributed to the corpus luteum hormone but which is now thought to be due to subthreshold amounts of estrogen.

Papanicolaou and Falk (19) and Falk and Papanicolaou (20) reported clitorine enlargement and changes in the skeletal musculature subsequent to the administration of APL to female guinea pigs. They attributed these masculinizing effects to hypertrophied interstitial tissue in the ovaries. Greene and Burrill (21) found that the administration of APL to juvenile female rats caused masculinization of the clitoris and suggested that the androgen produced by such treated ovaries might be progesterone. Desclin (22) reported that ovarian grafts, luteinized by APL, did not prevent castration atrophy of the male accessories in the rat. Marx and Bradbury (23) administered APL to suckling rats and correlated the masculinization of the external genitalia with hypertrophy of the thecal and interstitial tissues of the gonad.

The identity of the androgenic substances which derive from ovaries has not been ascertained. While the administration of large amounts of progesterone elicits masculinizing effects in certain laboratory rodents, the evidence seems to indicate that the ovaries may liberate another androgen which is similar to but not identical with that secreted by the testis. Deanesly (13) reported that gonadotropic extracts causing ex-

tensive luteinization of the granulosa did not alter appreciably the androgenicity of ovarian ear grafts in the rat. From studies upon the growth curves of male accessory glands, Hill and Strong (11) concluded that the physiological response induced by ovarian grafts in the ears is most nearly duplicated by the experimental administration of testosterone propionate plus an estrogen.

The two distinct types of atresia found in the present abnormal ovaries are suggestive of those described (24) in normal bats of the genus Myotis. The most distinctive histological feature of the pathological ovaries under consideration is the hypertrophy and persistence of the theca interna of vesicular follicles undergoing atresia and the absence of luteinized tissue. When these ovaries are transplanted to the kidneys of castrate male hosts, some luteinization of the theca interna does occur. The conspicuous accumulations of unluteinized theca interna in the intact gonads suggests that this histological change is associated with the capacity of the ovaries to exert an androgenic action. No evidence has been obtained regarding the source of the estrogen secreted by these glands.

An interpretation of the endocrine imbalance. The high incidence of pyosalpingitis and pyometria in the present abnormal rats suggests that the accessories have been subjected to a prolonged hyperestrogenic or hyperandrogenic state. Such inflammatory reactions in the tubes and uteri were uncommon among normal females of this strain. Unpublished observations by the author demonstrate that severe inflammatory reactions in the female accessories frequently occur in adult rats subsequent to the administration of estrogens or androgens early in life. Metaplasia of the uterine epithelium may or may not be present in the treated animals. Shay, Gershon-Cohen, Paschkis, and Fels (25) reported that testosterone propionate administered to young rats resulted in the production of luteal cysts in the ovaries of the adults. On the basis of their figures and their description of the abnormaltiy, it is the writer's opinion that these workers produced pyosalpingitis and pyometria rather than ovarian tumors of the luteal type. The ovary itself apparently escaped their attention since all of their sections appear to pass only through the inflamed tubes or uteri. In my own series of animals, it appears that the defective ovaries reflect an abnormal liberation of gonadotropic hormones by the hypophysis. Apparently, FSH is produced principally. As the result of continuous follicle stimulation and no luteinization, the sex accessories are subjected to prolonged estrogen stimulation. It is possible that the continued action of estrogen renders the tubes and uteri susceptible to bacterial infection, thus encouraging pyosalpingitis and pyometria. It has not been determined definitely whether the uterine metaplasia results directly from the pyometria or from the estrogen stimulation. However, the fact that squamous metaplasia appeared in the uteri of two animals in the absence of pyometria suggests that the metaplasia is attributable directly to prolonged estrogen stimulation.

Mild mucification of the vaginal epithelium was characteristic of the intact animals of this series. It is known that mucification may result from estrogen alone, androgen alone, or from estrogen modified by androgen. It has been demonstrated that the administration of estrogen in amounts insufficient to cornify the vaginal epithelium is capable of producing mucification (26, 27). Testosterone and certain related androgens have the property of mucifying the vaginae of oöphorectomized rats (18). Testosterone and androstanediol are capable furthermore of modifying the action of estrogen and thus of producing full mucification when administered with it (28, 29). Crystalline progesterone by itself does not mucify the vagina but it does modify the action of estrogen so that full mucification is obtained by giving the two hormones in conjunction (30, 31). If the estrogen output of the ovaries of the present abnormal animals is high enough to sensitize the uterus to bacterial infection, to produce uterine

metaplasia and cystic glandular hyperplasia, and to cause hypertrophy of the musculature of the seminal vesicles, it seems probable that this level of estrogen secretion would be too great to produce vaginal mucification. Since luteinized tissue seems to be absent in these gonads it is assumed that appreciable amounts of progesterone are not secreted. Considering these points, it seems justifiable at the present to attribute the vaginal mucification in these females to ovarian estrogen the action of which is modified by some ovarian androgen similar to testosterone or androstanediol. This concept is suggestive of the observations of Hill and Strong (11) who found that the straight growth curve of male accessories stimulated by testosterone propionate is caused to plateau by the addition of estrogen.

The defects characterizing the external genitalia of these 7 animals may be simulated experimentally in females by the prenatal administration of estrogen or by the administration of these compounds during the first few days of postnatal life (1, 2, 4, 32). Both Hain and Greene and collaborators have interpreted the cleft phallus as a hypotrophic effect of the estrogen, the two halves of the preputium being prevented from fusing. Turner and Burkhardt (4) suggested that this type of abnormal development might be accounted for on the basis of an androgen which produced cleavage of the preputium, enlargement of the glans clitoridis and a mild hypertrophy of the erectile tissues of the clitoris. The adult ovaries of these spontaneously abnormal females are androgenic as is demonstrated by the transplantation experiments here reported. It is known also that some of the ovaries from adult rats receiving estrogen early in life are capable of maintaining secretory activity in the ventral prostatic lobes when transplanted to the kidneys of male castrates (unpublished observations of Turner and Haffen). While the embryological differentiation of the preputium of the female must remain a contended point until more detailed studies are forthcoming, it is probable that an ovarian androgen accounts for the hypertrophy of the glans clitoridis and the cavernous tissues in the adult females possessing both the spontaneous and experimental defects of the external genitalia.

SUMMARY

A spontaneous ovarian defect in the rat is described and interpreted. All of the 7 females possessed a characteristic defect of the external genitalia, and there was a high incidence of pyosalpingitis and pyometria. The ovaries from these animals are hyperestrogenic and androgenic. Luteinized tissue was absent. The androgenicity of these ovaries is correlated with the hypertrophy and persistence of the theca interna of atretic ovarian follicles.

REFERENCES

1. HAIN, A. M.: *Quart. J. Exper. Physiol.* 25: 131. 1935.
2. GREENE, R. R.: *Proc. Soc. Exper. Biol. & Med.* 36: 503. 1937.
3. GREENE, R. R., AND A. C. IVY: *Science* 86: 200. 1937.
4. TURNER, C. D., AND W. L. BURKHARDT: *Proc. Soc. Exper. Biol. & Med.* 42: 267. 1939.
5. STEINACH, E., AND H. KUN: *Arch. f.d. ges. Physiol.* 227: 266. 1931.
6. LIPSCHÜTZ, A.: *Arch. f. Path. Anat.* 285: 35. 1932.
7. HILL, R. T.: *Endocrinology* 21: 495. 1937.
8. HILL, R. T.: *Endocrinology* 21: 633. 1937.
9. HILL, R. T., AND W. U. GARDNER: *Anat. Rec. Suppl.* 64: 21. 1936.
10. HILL, R. T. AND M. T. STRONG: *Endocrinology* 22: 663. 1938.
11. HILL, R. T., AND M. T. STRONG: *Endocrinology* 27: 79. 1940.
12. DEANESLY, R.: *J. Physiol.* 92: 34P. 1938.
13. DEANESLY, R.: *Proc. Roy. Soc. London, s. B.* 126: 122. 1938.
14. LAMAR, J. K.: *Anat. Rec. Suppl.* 70: 45. 1937.
15. GREENE, R. R., M. W. BURRILL AND A. C. IVY: *Endocrinology* 24: 351. 1939.
16. GREENE, R. R., M. W. BURRILL AND D. M. THOMSON: *Endocrinology* 27: 469. 1940.
17. NELSON, W. O.: *Anat. Rec. Suppl.* 67: 110. 1936.
18. DEANESLY, R., AND A. S. PARKES: *Quart. J. Exper. Physiol.* 26: 393. 1937.

19. Papanicolaou, G. N., and E. A. Falk: *Proc. Soc. Exper. Biol. & Med.* 31: 750. 1934.
20. Falk, E. A., and G. N. Papanicolaou: *Anat. Rec. Suppl.* 64: 16. 1936.
21. Greene, R. R., and M. W. Burrill: *Proc. Soc. Exper. Biol. & Med.* 40: 514. 1939.
22. Desclin, L.: *Compt. rend. Soc. de Biol.* 128: 557. 1938.
23. Marx, L., and J. T. Bradbury: *Anat. Rec.* 78: 79. 1940.
24. Guthrie, M. J., and K. R. Jeffers: *J. Morphol.* 62: 523. 1938.
25. Shay, H., J. Gershon-Cohen, K. E. Paschkis and S. S. Fels: *Endocrinology* 25:933. 1939.
26. Robson, J. M.: *J. Physiol.* 71: iii. 1931.
27. Allen, W. M., and R. K. Meyer: *Anat. Rec.* 61: 427. 1935.
28. Korenchevsky, V., and M. Dennison: *J. Pathol. & Bact.* 42: 91. 1936.
29. Korenchevsky, V., and M. Dennison: *J. Pathol. & Bact.* 43: 345. 1936.
30. Selye, H., J. S. L. Browne and J. B. Collip: *Proc. Soc. Exper. Biol. & Med.* 34: 198. 1936.
31. Selye, H., J. S. L. Browne and J. B. Collip: *Proc. Soc. Exper. Biol. & Med.* 34: 472. 1936.
32. Greene, R. R., M. W. Burrill and A. C. Ivy: *Proc. Soc. Exper. Biol. & Med.* 43: 32. 1940.

THE INCREASED ESTROGENIC POTENCY OF HUMAN URINE AFTER ZINC-HYDROCHLORIC ACID HYDROLYSIS[1]

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IN AN EARLY PUBLICATION (1), it was reported that boiling of urine under a reflux for 3 hours with 4 gm. % of zinc dust and 15 vol. % of HCl resulted in a marked increase in the yield of estrogenic potency over that acquired by the optimum conditions of simple acid hydrolysis, namely, 10-minute boiling under a reflux with 15 vol. % of HCl. The suggestion was made that better hydrolysis and conversion of estrone to estradiol might explain the augmented potency and that the ratio of total estrogen after Zn-HCl hydrolysis (T_{zn}) to total estrogen after simple HCl hydrolysis (T_o) might provide an index of the relative estrone content of urines. Further experimentation revealed, however, that neither estrone conversion nor increased hydrolysis could account for the very high T_{zn} to T_o ratios observed in certain urines and it was concluded (2) that non-estrogenic compounds closely related to the estrogens (possibly estrogen break-down products) were rendered estrogenic by Zn-HCl hydrolysis. The object of this paper is to report in more detail our findings with the use of Zn-HCl hydrolysis and the evidence therefrom that T_{zn} to T_o ratios provide a relative index of estrogen destruction in the body.

Effect of Zn-HCl *Hydrolysis Upon Crystalline Estrogens*

Estrone is the most labile of the naturally occurring estrogens. It is especially sensitive to oxidation and in watery solution its estrogenic activity[2] is rapidly destroyed by acid and heat; that is, 50% destruction in 20 minutes at 100° C. with 15 vol. % HCl. (Ten minutes at 100° C. with 15 vol. % HCl causes no loss in the acitivity of estrone, estriol or estradiol.) Even in the presence of an hydrogen liberator, there is an 80 to 90 % loss of potency when crystalline estrone in distilled water is subjected to the 3-hour heating period of Zn-HCl hydrolysis. In urine, however, crystalline estrone is not as labile to heat and acid. Twenty minutes at 100° C. with 15 vol. % HCl causes no loss in the potency of estrone added to urine.[3] Zn-HCl hydrolysis of crystalline estrone added to urine yields 4 to 7 times as much activity as that present in the original estrone. The highest yields are acquired when small amounts of estrone are added to large volumes of urine (table 1), the optimum concentration being no more than 40 R.U. (20 to 25 γ) per 100 cc. It has been found that

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[1] From the Mrs. William Lowell Putnam Investigation of the Toxemias of Pregnancy, aided by grants from the Committee for Research in Problems of Sex, National Research Council.
[2] All estrogen values in this investigation and in other recent publications by the writers have been acquired by a standardized method of bioassay which is being separately reported.
[3] In all experiments where crystalline estrogens have been added to urine, the T_o and T_{zn} values of the urine itself have, of course, been allowed for in calculating the effect upon the added estrogens. In some experiments, urine was used from which all estrogens present after Zn-HCl hydrolysis had been removed by 24-hour extraction with benzene.

a concentrated phosphate buffer solution (69 gm. NaH_2PO_4 and 179 gm. Na_2HPO_4 per liter) provides a fairly satisfactory substitute for urine. Twenty minutes at 100° C. with 15 vol. % HCl causes no loss in the potency of estrone added to this solvent. Zn-HCl hydrolysis of estrone in concentrated phosphate buffer yields 3 to 4 times as much potency as was present in the original estrone. Here again the largest yields are with low concentrations of estrone, but even under what appear to be optimum conditions, that is, no more than 30 R.U. (15 to 20 γ) of estrone per 100 cc. of solvent, the yield is only half that from Zn-HCl hydrolysis of estrone in urine at the optimum concentration. The lower yields in phosphate buffer and in more concentrated urine solutions are not due to incomplete conversion of estrone, since semicarbazide treatment[4] has demonstrated the complete disappearance of this hormone after Zn-HCl hydrolysis under all of the conditions tabulated in table 1. It seems probable, therefore, that the high salt content of urine and of the buffer solution increases the solubility of the added estrogen, thereby hastening the reaction involved

TABLE 1. EFFECT OF SOLVENT UPON Zn-HCl HYDROLYSIS OF ESTRONE

Distilled water					Urine of normal women					Concentrated phosphate buffer solution				
Estrone added	Volume of solvent		Potency[1] after Zn-HCl		Estrone added	Volume of solvent		Potency[1] after Zn-HCl		Estrone added	Volume of solvent		Potency[1] after Zn-HCl	
R.U.	cc.	R.U. / 1 cc.	R.U.	% loss	R.U.	cc.	R.U. / 1 cc.	R.U.	% gain	R.U.	cc.	R.U. / 1 cc.	R.U.	% gain
120	10	12	24	80	15	10	1.5	60	300	120	100	1.2	360	200
60	20	3.0	<8	>86	150	250	0.6	750	400	150	250	0.6	450	200
200	200	1.0	20	90	60	100	0.6	300	400	200	400	0.5	600	200
60	100	0.6	10	83	20	50	0.4	140	600	60	200	0.3	250	315
60	200	0.3	10	83	20	100	0.2	140	600	15	100	0.15	60	300

[1] Extracted 24 hours with benzene and taken up in olive oil.

and preventing destruction, and that the lower yields are due to estrone destruction prior to its conversion into some compound of greater estrogenic potency. The activity of crystalline estriol and α estradiol is unaffected by Zn-HCl hydrolysis, whether performed in urine or phosphate buffer.

Hydrogenation with zinc and acid might well convert the ketonic form to the more potent α estradiol. In this case one would expect, with our method of assay, a 900% increase in potency. The highest increase observed, with urine as a solvent, was 600%, but from the results discussed above it seems possible that some loss of estrone prior to its conversion by Zn-HCl hydrolysis may take place even under optimum conditions, accounting for the failure to get the theoretical yield. Another possibility is that β as well as α estradiol is formed (3). Since the β form is low in estrogenic activity, its presence would lower the potency of the final product. In either event, the following experiments indicate that Zn-HCl hydrolysis of estrone converts it into estradiol and suggest that this compound is practically, if not entirely the only estrogen produced. (*a*) Recovery experiments (2) have shown that α estradiol is almost quantitatively taken into the weakly acidic estrone fraction by our modification of the Cohen and Marrian separation of estrogens. All but about 10% of the activity of Zn-HCl hydrolyzed estrone, whether urine or concentrated phosphate buffer is used as a medium, is also recovered in the estrone fraction. (*b*)

[4] The technic of semicarbazide treatment was that recommended by E. A. Doisy in a personal communication. For a description, see reference 2.

The weakly acidic fraction from 500 γ of Zn-HCl hydrolyzed estrone (using one liter of concentrated phosphate buffer as solvent) gave, with concentrated H_2SO_4, the strong sapphire blue fluorescence specific for estradiol. We were unable, due to unavoidable contamination with zinc salts, to obtain a color test with the strongly acidic estriol fraction of this experiment. Both David's estriol reagent (4) and concentrated H_2SO_4 gave a yellow coloration only. We cannot say, therefore, whether the 10% of activity in the strongly acidic estriol fraction is due to a small amount of estradiol carried over into this fraction or to some estrogen other than estradiol.

Effect of Zn-HCl *Hydrolysis Upon the Urine of Women*

The potency of the estrone and estriol fractions of three urines from normally pregnant women after HCl hydrolysis was compared with the potency of these fractions after Zn-HCl hydrolysis of the specimens (table 2). In all three the increased activity was divided approximately in a 2:1 ratio between the two fractions. The

TABLE 2. EFFECT OF Zn-HCl HYDROLYSIS ON URINES FROM PREGNANT WOMEN (Comparison of Potencies of Fractions after HCl Hydrolysis with Those after Zn-HCl Hydrolysis, Demonstrating the Partition of the Increased Estrogenic Activity between the Weakly and Strongly Acidic Phenols)

	HCl hydrolysis, modified Cohen & Marrian separation (2)			Zn-HCl hydrolysis, modified Cohen & Marrian separation (2)			Difference—Amount of increase from Zn-HCl hydrolysis				
	Estrone fraction	Estriol fraction	T_o	Estrone fraction	Estriol fraction	T_{an}	Total	Estrone fraction		Estriol fraction	
	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	%	R.U./24°	%
Mrs. L. 3 mo. pregnant	1,450	1,450	2,900	3,900	2,900	6,800	3,900	2,450	63	1,450	37
Mrs. B. 8½ mo. pregnant	2,900	58,000	60,900	54,000	81,000	135,000	74,100	51,100	69	23,000	31
Mrs. DeP. 8 mo. pregnant	3,000	26,700	29,700	50,000	56,000	106,000	76,300	47,000	62	29,300	38

increase in the estriol fraction, according to the results with crystalline estrone, could not be accountable to estrone conversion. Furthermore, in the two late pregnancy urines, conversion of estrone into something 7 times as active could account for only part of the increased activity of the estrone fraction, even if all the original potency of the estrone fraction were accountable to estrone. We now know that at least half of the potency of estrone fractions of all late pregnancy urines is due to the presence of α estradiol (5, 6). Conversion of estrone, therefore, cannot be the only factor involved in the increased potency of late pregnancy urines after Zn-HCl hydrolysis. The same may be said of all urines from women, since the highest proportional increases in non-pregnancy as well as pregnancy have been found in specimens containing little or no estrone (table 4). Semicarbazide treatment of extracts of Zn-HCl hydrolyzed urine from both pregnant and non-pregnant women has not affected their estrogenic activity, indicating, as would be expected from the experiments with crystalline estrone, that no estrone is present after hydrogenation. The conclusion is, therefore, that Zn-HCl hydrolysis of urine not only converts estrone into estradiol but also either produces or frees some other estrogen not present in active form in HCl hydrolyzed urine, approximately one-third of which is strongly phenolic, that is, not estradiol.

With the object of acquiring information as to the possible sources, other than estrone conversion, of the increased estrogenic potency of Zn-CHl hydrolyzed specimens, the following experiments were performed upon 7 urines from women having normal menstrual cycles and 3 from pregnant women (table 3). Upon one portion of each urine the T_{sn} value was determined. The remainder of the specimen was submitted to HCl hydrolysis and extracted, a portion of the extract being used for assay

TABLE 3. Zn-HCl HYDROLYSIS OF URINE, OF RESIDUAL URINE AFTER T_o EXTRACTION, AND OF T_o EXTRACTS, INDICATING THE POSSIBLE SOURCES OF THE INCREASED POTENCY WITH ZINC

Urine specimen	HCl hydrolysis		Zn-HCl hydrolysis		T_{sn} minus T_o	Zn-HCl on T_o residual		Zn-HCl on T_o extract		
	Ether extract[1] T_o	Benzene extract[2] T_o	Ether extract[1] T_{sn}	Benzene extract[2] T_{sn}		Benzene extract,[2] total	% of T_{sn} minus T_o	Benzene extract,[2] total	Total minus T_o value	% of T_{sn} minus T_o
	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	R.U./24°	%	R.U./24°	R.U./24°	%
Mixed—luteal phase of normal cycles		45		105	60	50	83	—	—	—
H.S. —18th to 20th day of normal cycle		134		370	236	200	85	150	16	6.7
S.W. —16th to 18th day of normal cycle	100		330		230	200	87	120	20	8.7
S.W. —19th to 21st day of normal cycle		93		300	207	185	89	—	—	—
S.W. —3rd to 5th day of normal cycle-menstruating		25		200	175	74	42	90	65	37
S.W. —1st to 3rd day of normal cycle-menstruating		20		133	113	33	29	77	57	50
	25		100		75	25	33	60	35	47
S.W. —2nd to 4th day of normal cycle-menstruating		10		85	75	34	45	36	26	35
E.B.F.—3 mo. pregnant normal	2,900		6,800		3,900	1,800	46	4,400	1,500	38
L.R. —8 mo. pregnant normal		78,000		155,000	77,000	19,500	25	130,000	52,000	68
D.P. —8 mo. pregnant diabetes, essential hypertension	31,700		106,600		74,900	16,000	21.5	77,000	45,300	61

[1] Shaken 4 times in a separatory funnel with 1/5th volume of ether.
[2] Extracted 24 hours in a continuous benzene extractor.

of the T_o value and the rest evaporated to dryness, taken up in concentrated phosphate buffer solution, hydrolyzed with zinc and HCl, and re-extracted. The residual acid urine (from which all estrogens free after simple HCl hydrolysis had been removed) was boiled for 3 hours under a reflux condenser with 4 gm. % of zinc dust and then re-extracted. Any increase from Zn-HCl hydrolysis of the residual urines after extraction of T_o or from Zn-HCl hydrolysis of the extracts of HCl hydrolyzed urines could not be accountable to decreased destruction of urinary estrogens in the presence of zinc, since, with both, HCl hydrolysis was performed prior to Zn-HCl hydrolysis. Any increase from Zn-HCl hydrolysis of the residual urines after HCl hydrolysis and extraction might be due to enhanced hydrolysis or to the conversion of benzene or ether-insoluble non-estrogenic compounds into benzene-soluble estrogens. Any increase from Zn-HCl hydrolysis of the extracts of HCl hydrolyzed urines could not be due to increased hydrolysis, since conjugated urinary estrogens are insoluble in the solvents used, as demonstrated by the fact that HCl hydrolysis (in concentrated phosphate solution) of benzene or ether extracts of unhydrolyzed urines

results in no increased potency (unpublished data), Zn-HCl hydrolysis of extracts of HCl hydrolyzed specimens would convert any estrone present into a compound of greater activity, probably estradiol, and might also convert certain non-estrogenic compounds soluble in benzene or ether into estrogens.

From the results on the 4 urines collected during the luteal phase of normal cycles,

TABLE 4. REPRESENTATIVE RESULTS DEMONSTRATING THE RELATION OF T_{zn} TO T_o RATIOS IN URINE TO THE METABOLISM OF ESTROGENS AS INDICATED BY THE PARTITION OF URINARY ESTROGENS

Donor	Urine specimen		HCl hydrolysis—Separated ether extract[1]				T_{zn} Total	T_{zn}/T_o
	Clinical condition	Comments	Estrone R.U./24° (R.U.×0.5=γ)	Estradiol R.U./24° (R.U.×0.05=γ)	Estriol R.U./24° (R.U.×0.5=γ)	Total R.U./24°	R.U./24°	
S.W.[2]	Normal cycle—days 1–3	Menstruation	0	14	7	21	140	6.7
	Normal cycle—days 7–8	Follicular phase	8	21	14	43	140	3.3
	Normal cycle—days 14–15	Luteal phase	21	32	53	106	270	2.5
	Normal cycle—days 20–21	Luteal phase	28	42	53	123	340	2.7
	Normal cycle—days 26–28	Last 3 days before ctma.	4.0	9.0	7	20	60	3.0
	Normal cycle—days 1–3	Menstruating	0	13	7	20	150	7.5
B. Van A.[2]	Dysfunctional flow	Flowing—no $P_{\frac{1}{2}}$	0	100	0	100	600	6.0
		After 30 mg. Progesterone	20	17	30	67	250	3.7
T.L.[2]	Dysfunctional flow	Flowing—no $P_{\frac{1}{2}}$	0	44	6	50	250	5.0
E.B.	Male—teratoma testis	After radiation	0	105	39	144	1,740	12.1
P.P.	Normal pregnancy	8½ months	800	2,500	25,000	28,300	85,000	3.0
		During labor	0	3,600	10,000	13,600	98,000	7.2
R.C.	Normal pregnancy	8½ months	3,300	6,700	53,000	63,000	106,000	1.7
		During labor	0	4,000	10,000	14,000	72,000	5.1
G.McC.	Clinically normal pregnancy prior to pre-eclamptic signs	8 months	330	1,000	5,000	6,330	30,000	4.7
	4 days later—sudden edema, rise in B.P. and albuminuria	No $P_{\frac{1}{2}}$	"	1,330	5,000	6,330	40,000	6.3
	2 wk. later—no edema, still high B.P. No alb.	After estrogen-progestin $P_{\frac{1}{2}}$[3]	1,330	1,330	10,000	12,700	25,000	2.0
J.E.	Pre-eclamptic toxemia. Mild signs. Alb. 1.3 gm./24°	8 months	340	1,330	8,000	9,670	37,000	3.8
	1 wk. later—severe signs. Alb. 2.6 gm./24°	No $P_{\frac{1}{2}}$	"	1,500	5,000	6,500	37,000	5.7
	1 wk. later—mild signs. Alb. 1.5 gm./24°	After estrogen-progestin $P_{\frac{1}{2}}$[3]	1,330	670	20,000	22,000	40,000	1.8
F.D.	Pre-eclamptic toxemia. Severe signs. Alb. 9.0 gm./24°	8 months. No $P_{\frac{1}{2}}$	0	670	2,700	3,370	16,700	5.0
	10 days later. Clinically no better Alb. 8.6 gm./24°	After estrogen-progestin $P_{\frac{1}{2}}$[3]	"	2,000	2,000	4,000	21,500	5.3

[1] For method (modification of Cohen and Marrian(10) see reference 2.

[2] In the study of urines of low estrogen content, 48 to 72-hour collections were extracted in order to secure enough estrogen for bioassay. For the sake of comparison, however, all results are expressed in terms of 24-hour excretion.

[3] For details of this $P_{\frac{1}{2}}$ in pre-eclampsia, see reference 5. The daily dosage in these particular cases was 10 mg. of estradiol benzoate (60,000 R.U. by our assay) and 20 to 40 mg. of progesterone.

one might conclude that better hydrolysis accounted for practically all of the difference between T_{zn} and T_o values, since 83 to 89% was recovered by zinc hydrolysis of the residuals from T_o extraction. However, with the urines collected during menstruation and pregnancy, less than half of the difference between T_{zn} and T_o values could be due to increased hydrolysis of conjugated estrogens. Thirty-five to 68% of the difference was gained from Zn-HCl hydrolysis of T_o extracts and must have been accountable either to estrone conversion or to estrogen manufacture from benzene or ether-soluble non-estrogenic substances. Urines collected at the time of menstruation contain no demonstrable estrone (table 4, unpublished data, and reference 7), and the

range of estrone contents of pregnancy urines (5) is considerably less than could account for the increased potency observed after hydrogenation of these extracts of HCl hydrolyzed urines. At the time of menstruation and during pregnancy, therefore, conversion of non-estrogenic urinary constituents into some compound or compounds having estrogenic potency must account for a fairly large percentage of the difference between T_o and T_{zn} values.[5] It seems probable that during the luteal phase of normal cycles also such conversion enters into the increase with Zn-HCl hydrolysis, but that at this time any non-estrogenic urinary constituents convertible by hydrogenation into estrogens are conjugated in a more stable form so that they are not freed by 10 minutes boiling with HCl, but are freed without destruction[6] by 3 hours boiling under a reflux with zinc and HCl.

It will be noted in table 3 that the values for Zn-HCl hydrolysis of residuals and of extracts do not add up to the total difference between T_o and T_{zn} of the urines themselves. Percentage figures based on bioassay can be only approximate, but the consistent loss, which amounted to 20% in the urines collected during menstruation, makes the observation seem significant. This loss might be due to destruction of urinary estrogens, other than estrone, estriol and estradiol, by HCl hydrolysis. Even if this were the whole explanation, such destruction would account for very little of the difference between T_{zn} and T_o values. Another possibility is the destruction during HCl hydrolysis of certain labile non-estrogenic substances which are protected and converted into estrogens in the presence of zinc. A very possible source of loss, and one which would be apparent only in the urines collected during menstruation and pregnancy, is in Zn-HCl hydrolysis of T_o extracts, this procedure being carried out in concentrated phosphate solution rather than urine itself (see section above on Zn-HCl hydrolysis of crystalline estrogens).

There is reason to suppose that estrogen destruction in the body is an oxidative process (8, 9). The proportion of the total difference between T_{zn} and T_o values, therefore, which is accountable to conversion of non-estrogenic urinary constituents into estrogens may well be due, at least in part, to rehydrogenation of estrogen breakdown products. From studies of estrogen metabolism in women (5, 7), we have concluded that decreased metabolic conversion of estrone to estriol, due to progestin deficiency, is accompanied by a reversal of the estradiol to estrone reaction and more rapid destruction of all estrogens, particularly estrone. A decrease of urinary estriol, therefore, together with a rise in estradiol and a drop or complete disappearance of demonstrable estrone (estrogenic potency of weakly acidic phenolic fractions removable by semicarbazide treatment), has been interpreted as signifying that destruction of estrogens, rather than metabolic conversion, is occurring. Such destruction appears to play an important role in normal and pathological processes and it is at these times that the highest T_{zn} to T_o ratios are found. (For representative data, see table 4.) It is also at these times that estrone conversion or increased hydrolysis must contribute only a part of the increase observed upon Zn-HCl hydrolysis. It seems more than likely, therefore, that the highest proportional increases of T_{zn} over T_o are due in large part to rehydrogenation of oxidized estrogen breakdown products back to material with estrogenic activity and that the ratios of T_{zn} to T_o provide a relative index

[5] Marrian (8) has suggested that Zn-HCl hydrolysis might convert a non-estrogenic urinary diketone into estriol. From the observations in table 2, the final product cannot be more than one-third estriol. Furthermore, with one urine of late pregnancy, Dr. Gregory Pincus was able to perform for us a David test (4) upon the estriol fraction of an HCl hydrolyzed portion of the specimen and upon the estriol fraction of a Zn-HCl hydrolyzed portion. The same value was obtained with both. Since this test is highly specific and quantitative for estriol, the result demonstrated that in this particular urine Zn-HCl hydrolysis had neither produced nor freed additional estriol.

[6] It is to be remembered that in comparing T_{zn} and T_o values we are comparing a 3-hour period of boiling in thepresence of zinc and acid with a 10-minute period of boiling with acid alone. Zinc does, of course, protect estrogens from being destroyed over the longer heating period used in Zn-HCl hydrolysis, probably by converting estrone to a more active reduced form and preventing oxidation of all estrogens.

of estrogen destruction in the body. Not all destroyed estrogen, however, is recoverable by Zn-HCl hydrolysis, as indicated by the fact that T_{zn} values after estrogen administration seldom approach the total amount of estrogen administered. (For representative data, see table 4.)

SUMMARY

The addition of powdered zinc to urine during hydrolysis by boiling with HCl yields 1.5 to 12 times as much estrogenic potency as may be obtained under the optimum conditions of hydrolysis with HCl alone.

Experiments on the three estrogens known to be present in human urine have revealed that Zn-HCl hydrolysis does not affect the potency of crystalline estradiol or estriol, but, in the proper medium, completely converts any estrone that escapes destruction into some product of greater activity. Estradiol is produced from Zn-HCl hydrolyzed estrone, and may be the only end product.

Experiments on Zn-HCl hydrolyzed urine have revealed that estrone conversion cannot be the only source of the increased potency observed, since the end product of Zn-HCl hydrolyzed urine differs both qualitatively and quantitatively from the end product of Zn-HCl hydrolyzed estrone.

Experiments aimed towards determining the possible sources, other than estrone conversion, of the difference between total estrogen after Zn-HCl hydrolysis and total estrogen after HCl hydrolysis have indicated that decreased destruction of urinary estrogens plays little if any part; that enhanced hydrolysis of conjugated estrogens may contribute; that better hydrolysis and estrone conversion could account for less than half of the total increase observed in certain urines and that a large part of the highest proportional increases is derived from non-estrogenic urinary constituents which are rendered estrogenic by Zn-HCl hydrolysis.

Studies on estrogen metabolism in women have demonstrated that the highest ratios of total estrogen after Zn-HCl hydrolysis to total estrogen after HCl hydrolysis are found in specimens in which the partition of the urinary estrogens reflects estrogen destruction. These high ratios may well be accountable to rehydrogenation of oxidized estrogen breakdown products.

Zn-HCl hydrolysis is not suitable for the study of urinary estrogens as such, and, if used exclusively, would lead to entirely misleading conclusions concerning estrogen metabolism.

The ratio of total estrogen after Zn-HCl hydrolysis to total estrogen after simple HCl hydrolysis appears to provide a relative index of estrogen destruction in the body.

We are indebted to Miss Sara Schiller for technical assistance.

REFERENCES

1. Smith G. V., and O. W. Smith: *Proc. Soc. Exper. Biol. & Med.* 36: 460. 1937.
2. Smith, O. W., G. V. Smith and S. Schiller: *Endocrinology* 25: 509. 1939.
3. Schwenk, E., and F. Hildebrandt: *Naturwiss.* 21: 177. 1933.
4. David, K.: *Acta brev. Neerland.* 4: 64. 1934.
5. Smith, G. V., and O. W. Smith: *Am. J. Obst. & Gynec.* 39: 505. 1940.
6. Huffman, M. N., D. W. MacCorquodale, S. A. Thayer, E. A. Doisy, G. V. Smith and O. W. Smith: *J. Biol. Chem.* 134: 591. 1940.
7. Smith, G. V., and O. W. Smith: *Am. J. Obst. & Gynec.* 36: 769. 1938.
8. Marrian, G. F.: *The Harvey Lectures*, Series 34: 37. 1939.
9. Heller, C. G.: *Endocrinology* 26: 619. 1940.
10. Cohen, S. L., and G. F. Marrian: *Biochem. J.* 28: 1603. 1934

THE MOUSE UTERINE WEIGHT METHOD FOR THE ASSAY OF ESTROGENS[1]

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IN SOME OF THE EARLIEST STUDIES on ovarian hormones the assay methods were based on the growth of the uteri in normal or castrated immature rabbits, rats, or mice (1, 2). No detailed study of this reaction in normal immature rats was made until Astwood and others (3, 4) determined the most favorable experimental conditions for a sensitive estrogen test based on an increase in uterine weight. They showed that this method is much more sensitive than the spayed rat vaginal smear method. However, the uterine weight response in the immature albino mouse to gonadotropins is much more uniform and more sensitive than in the rat (5–9). These favorable reactions led us to investigate the mouse uterine weight response to pure estrogens, androgens, progesterone, and urine and blood extracts. Von Haam (9) used the mouse uterine response for estrogens in urine extracts, but his results cannot be compared with ours because he does not give his methods and results in sufficient detail. The paper by B. B. Rubinstein and David Duncan which appears in this issue of *Endocrinology* is based in part on our method. In a subsequent paper we shall give data obtained by the application of this method to blood and urine extracts. In the present paper we present our results on pure steroid hormones.

PREPARATIONS AND PROCEDURE

We are indebted to Dr. George F. Cartland of the Upjohn Company for the specially purified estrone, equilin and estriol; to Professor O. Wintersteiner for the α-estradiol-diacetate, α-estradiol, β-estradiol, β-estradiol-3-benzoate, β-estradiol-diacetate, 7-keto-estrone, equilin, and β-dihydroequilenin; to Dr. Erwin Schwenk of the Schering Corporation for the α-estradiol, α-estradiol benzoate, α-estradiol-dipropionate, β-estradiol, Δ_6-equilin, equilin, α-dihydroequilin, progesterone, testosterone propionate, testosterone, dehydroisoandrosterone, and androsterone; and to Dr. J. A. Morrell of Squibb and Sons for the stilbestrol. The products received dissolved in oil were equilin (S), β-estradiol monobenzoate (S) from estrone, β-estradiol (S) from mares' urine, β-estradiol (S) from estrone, α-dihydroequilin and Δ_6-equilin. All other substances were received in crystalline form. The letters in parentheses (C), (S), (W), on the graphs indicate that the preparations with these designations came from Doctors Cartland, Schwenk, or Wintersteiner, respectively.

After a number of preliminary trials we adopted the following routine procedure. Immature female albino mice (from Maple Grove Rabbitry, Springfield, Missouri), weighing 6 to 8 gm. each, were injected subcutaneously twice daily with 0.05 cc.

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of the sesame oil solution of hormone for 3 successive days. Five animals were used on each level and 18 hours after the last injection the animals were killed and the carefully dissected uteri weighed on a sensitive torsion balance. In parallel with assays for any given date, 5 or more controls were injected with sesame oil only and 2 or more groups of 5 mice each were injected with pure estrone in sesame oil at several levels. All samples including the controls and standard estrone solutions were issued to one of us as unknowns.

EXPERIMENTAL RESULTS

In figures 1 to 5, the increases in the weights of uteri over the control weights are plotted against increasing doses of hormones. In figure 1 the curve for estrone is drawn through the average values obtained from the number of animals indicated. The variations in uterine responses to estrone are indicated by the vertical lines. It is evident that α-estradiol and stilbestrol are practically the same in activity by this test and that estriol is very low in activity compared to estrone. It is of particular tnterest that the types of curves obtained for estrone and estriol are similar to those obtained for uterine weight responses to these estrogens in the immature rat (10).

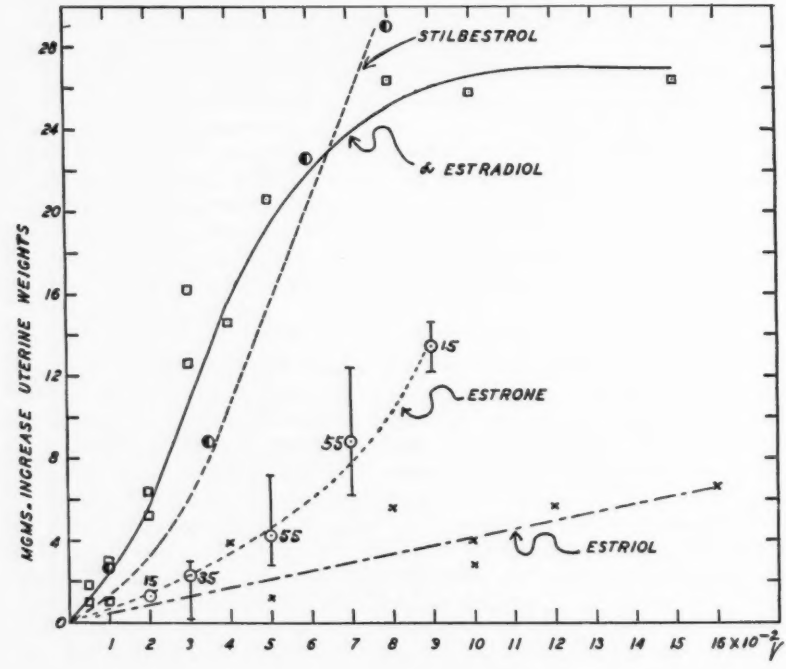

Fig. 1

Comparison of α-estradiol, α-estradiol monobenzoate, α-estradiol dipropionate, and α-estradiol diacetate in figure 2 shows that the dipropionate is relatively less potent than the other three estrogens. It is possible that the dipropionate may have been contaminated by β-estradiol dipropionate. The other esters possess very nearly the same activity per equivalent weight of α-estradiol as the free α-estradiol itself.

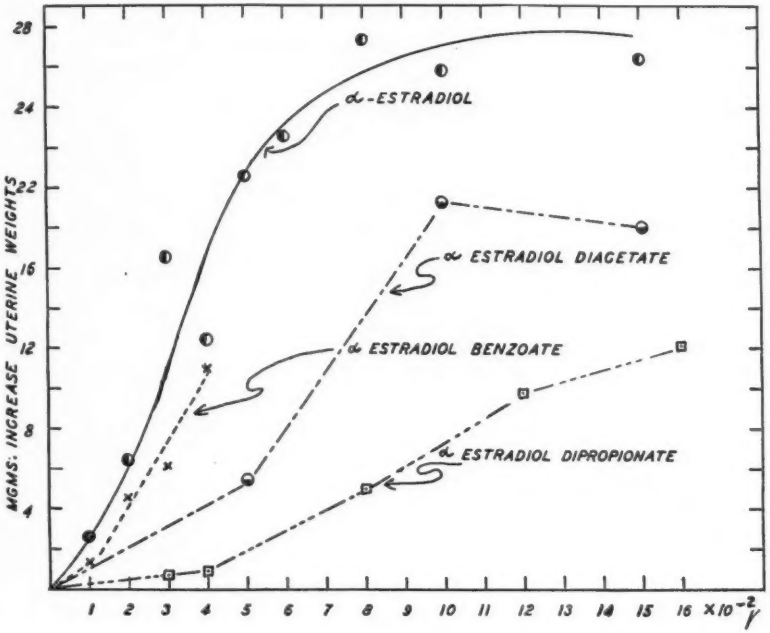
MGMS. INCREASE UTERINE WEIGHTS
α-ESTRADIOL
α ESTRADIOL DIACETATE
α ESTRADIOL BENZOATE
α ESTRADIOL DIPROPIONATE
γ

Fig. 2

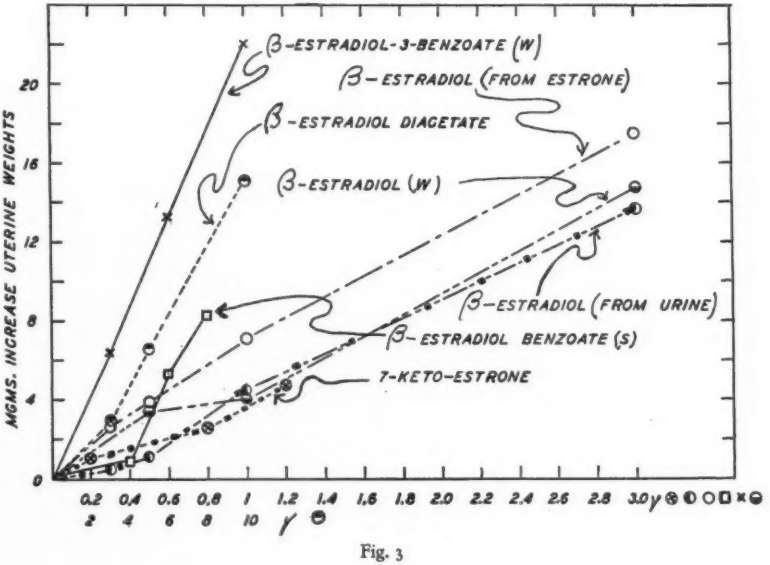
MGMS. INCREASE UTERINE WEIGHTS
β-ESTRADIOL-3-BENZOATE (W)
β-ESTRADIOL (FROM ESTRONE)
β-ESTRADIOL DIACETATE
β-ESTRADIOL (W)
β-ESTRADIOL (FROM URINE)
β-ESTRADIOL BENZOATE (S)
7-KETO-ESTRONE
γ

Fig. 3

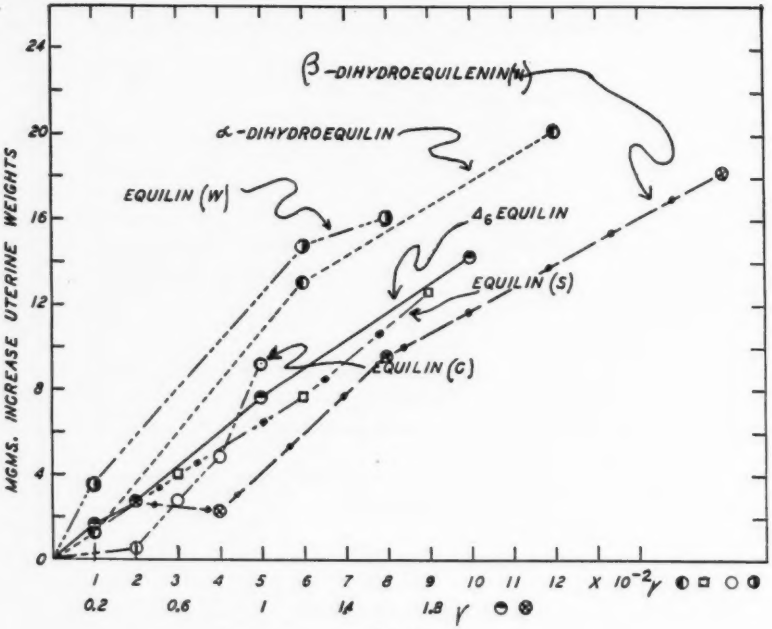
β-DIHYDROEQUILENIN(W)
α-DIHYDROEQUILIN
EQUILIN (W)
Δ6 EQUILIN
EQUILIN (S)
EQUILIN (G)
MGMS. INCREASE UTERINE WEIGHTS
24
20
16
12
8
4
0
1 2 3 4 5 6 7 8 9 10 11 12 X 10⁻² γ
0.2 0.6 1 1.4 1.8 γ

Fig. 4

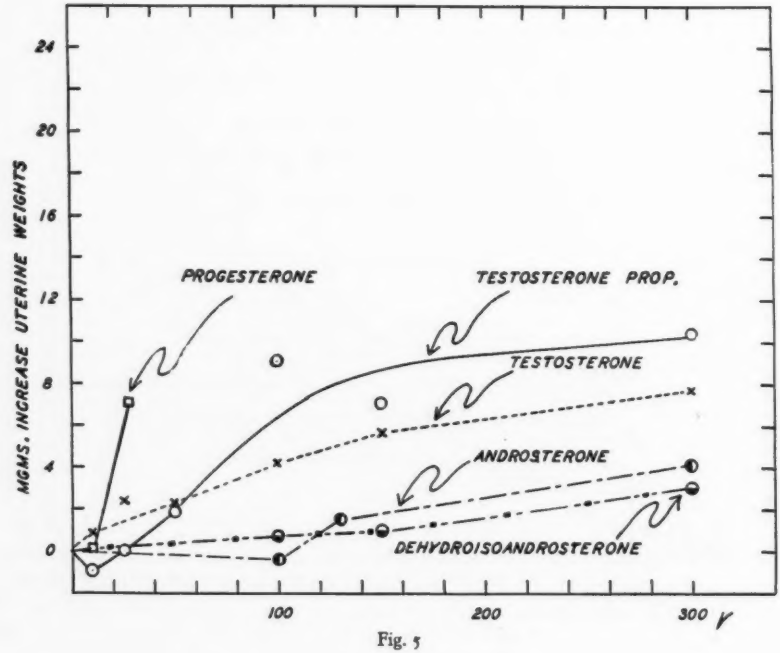
PROGESTERONE
TESTOSTERONE PROP.
TESTOSTERONE
ANDROSTERONE
DEHYDROISOANDROSTERONE
MGMS. INCREASE UTERINE WEIGHTS
24
20
16
12
8
4
0
100
200
300 γ

Fig. 5

Comparison of figure 2 and figure 3 shows that β-estradiol and its esters are of a low order of activity compared with the α series. The remarkable difference in the two samples of β-estradiol-3-benzoate suggests that the two preparations are not identical. This may involve in part isomerism on carbon 17 or different positions of the benzoyl group. It should be noted that the β-estradiol-3-benzoate is much more potent even on the absolute weight basis than the free β-estradiol, whereas in the α series this is not true. It is evident that the partial oxidation of estrone to form the 7-keto-estrone lowers the activity greatly. On the other hand the β-estradiol diacetate is less than one-fortieth as active as the free β-estradiol.

In figure 4 the comparative results in the equilin series are given. It is evident that reduction of the 17-keto position as usual improves the potency. In fact, α-dihydroequilin as well as the two equilin samples are more potent than estrone. The

TABLE 1. THE EFFECT OF URINARY ANDROGENS ON THE UTERINE RESPONSE TO LOW LEVELS OF ESTRONE
(Five mice used for each group)

Group	Total dose of			Average uterine weight	Increase over control
	Estrone	Androsterone	Dehydroiso-androsterone		
	γ	γ	γ	mg.	
1	0	0	0	4.6	—
2	0.05	0	0	10.2	5.6
3	0.05	5	0	10.4	5.8
4	0.05	10	0	8.7	4.1
5	0	5	0	5.1	0.5
6	0	10	0	5.2	0.6
7	0.05	0	5	9.6	5.0
8	0.05	0	10	8.8	4.2
9	0	0	5	5.4	0.8
10	0	0	10	4.7	0.1
11	0.05	0	0	14.1	8.8
12	0.05	50	0	12.0	6.7
13	0	50	0	5.0	0
14	0.05	0	125	13.8	8.5
15	0	0	125	6.4	1.1
16	0	0	0	5.3	—

remarkable agreement in the values for Schering's and Doctor Cartland's equilin is worth noting. Shifting the double bond in equilin to position 6, 7 lowers the activity about 20-fold.

Although the androgens also stimulate the growth of the uteri the total dose required is 100γ or more for a marked response. Progesterone showed no effect at 10γ, but a good response at the 25γ level. Neither combinations of estrone plus progesterone injected concurrently, nor preliminary treatment with estrone followed by progesterone caused sufficiently significant histological changes in the uterus to enable us to distinguish estrone from progesterone action.

In table 1 are presented the uterine weight responses to 0.05γ estrone with and without androsterone or dehydroisoandrosterone in ratios in which they are likely to exist in extracts from human urine. It should be noted that experiments on groups 1 to 10, inclusive, were conducted at least thirteen months prior to those on groups 11 to 17. The differences in control weights and in increases in uterine weights illustrate how important it is to allow for seasonal, temperature, etc., variations by including controls and standards in each assay. It is evident that there is a suggestion of a slightly antagonistic action of the androgens at the levels used toward a standard of 0.05γ estrone. The difference is, however, negligible.

TABLE 2. RELATIVE ACTIVITIES OF ESTROGENS BY THE UTERINE MOUSE METHOD BASED ON A 6 MG. INCREASE IN UTERINE WEIGHT AND TAKING ESTRONE ARBITRARILY AT 100% ACTIVITY

	Activity
Estrone	100
Estriol	40
α-Estradiol	300
Stilbestrol	250
α-Estradiol diacetate	110
α-Estradiol benzoate	200
α-Estradiol (dipropionate)	70
β-Estradiol (from estrone)	7.5
β-Estradiol (from mare urine)	5
β-Estradiol-3-benzoate (W)	20
β-Estradiol benzoate (S)	10
β-Estradiol diacetate	0.1
Equilin (S)	110
Equilin (C)	110
Δ_6-Equilin	7.5
α-Dihydroequilin	200
β-Dihydroequilenin (W)	6
Progesterone	0.2
Testosterone propionate	0.07
Testosterone	0.03
Androsterone	<0.01
Dehydroisoandrosterone	<0.01

Table 2 gives the relative activities of the steroid studied as compared with estrone taken as 100%.

SUMMARY

The sensitivity and relative consistency of uterine weight responses in immature albino mice makes this assay method a very reliable one providing all assays are made in parallel with controls and several levels of estrone standards and also providing that the extracts are prepared free from gonadotropins. Availability of the animals at low cost also is an important item. In the preparation of urine or blood extracts it may be best to separate the phenolic estrogens from the neutral androgens, but for most comparative studies the amounts of androgens likely to be present are too low to interfere seriously. The method may prove to be of some value in studying non-phenolic estrogens or in quantitative studies on the separation of phenolic estrogens from progesterone.

REFERENCES

1. BÜLBRING, E., AND J. H. BURN: *J. Physiol.* 85: 320. 1935.
2. BROWNLEE, G.: *Quart. J. Pharm. & Pharmacol.* 11: 11. 1938.
3. ASTWOOD, E. B.: *Endocrinology* 23: 25. 1938.
4. LAUSON, H. D., C. G. HELLER, J. B. GOLDEN AND E. L. SEVRINGHAUS: *Endocrinology* 24: 35. 1939.
5. HAMBURGER, C., AND K. PEDERSEN-BJERGAARD: *Quart. J. Pharm. & Pharmacol.* 10: 662. 1937.
6. LEVIN, L., AND H. H. TYNDALE: *Endocrinology* 21: 619. 1937.
7. EVANS, J. S., L. HINES, R. VARNEY AND F. C. KOCH: *Endocrinology.* 26: 1005. 1940.
8. EVANS, J. S., L. HINES, J. J. CEITHAML AND F. C. KOCH: *Endocrinology* 26: 1012. 1940.
9. VON HAAM, E.: *Am. J. Clin. Path.* 10: 205. 1940.
10. DORFMAN, R. I., T. F. GALLAGHER AND F. C. KOCH: *Endocrinology* 19: 33. 1935.

LOCAL RESPONSES OF THE 'SEXUAL SKIN' AND MAMMARY GLANDS OF MONKEYS TO CUTANEOUS APPLICATIONS OF ESTROGEN

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THE EFFECTIVENESS of estrogen administered cutaneously in creams, oils, alcohol or benzene has been clearly demonstrated (1–4). This treatment has usually been directed toward systemic responses. Some experiments have demonstrated that estrogen may act locally on breast tissue or on vaginal epithelium without affecting other more distant organs. In the case of the vaginal epithelium extremely small amounts of estrogen were effective (5).

The direct application of estrogen, when possible, may prove desirable under certain conditions where systemic reactions are not wanted; for instance, in atrophic diseases of the vulva after menopause, at levels below that sufficient for the uterine reaction to induce recurrence of menstruation. From the experimental viewpoint the localization of the response to subjacent tissues adds further evidence concerning the basic reactions to estrogen; i.e. whether direct or through intermediation of other endocrine glands. This is especially important because uterine and vaginal epithelium in tissue cultures has not yet been shown to react to estrogen (6).

The red and swollen condition of the 'sexual skin' of normal female monkeys develops progressively at the approach of puberty and is intensified during the follicular phase of the menstrual cycle. This reaction disappears after ovariectomy and can be induced again by injections of estrogens (7). Therefore, it offers an ideal test object for local response.

The mammary glands are also ideal for study of local reactions to estrogens. They react to very small amounts of hormone, although longer times may be required than for vaginal or uterine effects. For instance, enough estrogen is secreted by transplanted ovarian tumor tissue to induce in several weeks growth of the mammary rudiments in male mice of the same strain (8), while similar ovarian tissue transplanted to ovariectomized females may not secrete enough hormone to induce an estrous reaction in the vaginal epithelium. Furthermore, extensive studies of the mammary glands of mice of several inbred strains (9) and their reactions to hormone stimulation (especially in relation to cancer) had accumulated many specimens for comparison (10). A study of responses of mammary tissue to alcoholic solutions of estrone applied locally to the overlying skin of male mice has been reported (11).

In monkeys systemic estrogenic stimulation of growth of both nipples and duct and alveolar tissue had been studied extensively, both in ovariectomized females (7) and immature females and males (12). Therefore, local estrogenic stimulation of the mammary rudiments of young male monkeys was attempted by unilateral cutaneous

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application. The dose of estrogen in the females was kept well below the threshold for the uterine growth reaction which is followed by menstruation after hormone withdrawal (13). During the course of this work Harold Speert generously sent us a carbon of his preliminary report (14) on a similar study in the monkey which our work confirms.

MATERIALS

Six immature female monkeys (*Macaca rhesus*) weighing from 3.2 to 2.4 kg. were used for study of reactions of the 'sexual skin' (table 1). These animals showed no swelling or reddening of these regions at the beginning of the experiments. Estrone dissolved in sesame oil (1 mg. per cc.) was further dissolved in alcohol to contain 10γ per cc. The alcoholic solution was dropped on the skin once daily from a hypodermic syringe. The amount was so small that it dried quickly. The area treated

TABLE 1. LOCAL APPLICATION OF ESTRONE TO THE 'SEXUAL SKIN' OF IMMATURE FEMALE MONKEYS (1 to 3μg. daily for 12 or 8 days)

No. of Monkeys	Amt. estrone daily	Period treated		Color at end	
		R.S.	L.S.	R.S.	L.S.
	γ	*days*	*days*		
1	1	12	8	1–1.5	2.
2	2	12	8	1–1.5	1.5
3	3	12	8	1–1.5	1.5

R.S.—'sexual skin' of the right side.
L.S.—'sexual skin' of the left side.

approximated 4 cm. in diameter on the right buttock and upper thigh. Treatment of the right side was continued for 12 days and then shifted to the left side for 8 days. During these periods alcohol alone was applied to the skin of the opposite side as a specific control. Daily recordings of the color of the 'sexual skin' and the amount of swelling were made, using a relative range of values from 1 to 4 (table 1).

Estrone in alcoholic solution (30γ per cc.) was applied to the left nipples and surrounding skin of two young male monkeys weighing 2310 and 2096 gm. Three γ in 0.1 cc. of alcohol was applied daily to the left gland for 30 and 126 days. Daily applications of 0.1 cc. of alcohol were made on the right breast at the same time as control treatment. Measurements of the nipples were made at several times during the course of the experiment (table 2). At the end of the experiment the breasts and nipples were removed. The glands were stained, dissected and preserved as whole mounts after a section of tissue had been removed for detailed histological examination. Sections were taken through the center of the nipples.

OBSERVATIONS

The 'sexual skin' of the areas to which the alcoholic solution of estrone was applied first showed appearance of color from 3 to 5 days after the treatment started and the erythema increased throughout the period treated to attain values of 1 to 1.5 as compared with a possible maximum of 4 (table 1). The first evidence of swelling appeared on the fifth day and progressed throughout the period of treatment. In all animals the response was entirely limited to the side to which the estrogen was applied. The maximum attained in these experiments was only 1.5 or 2 as rated in the scale of 1 to 4 used as standards.

During the second half of the experiment, when the treated and control sides

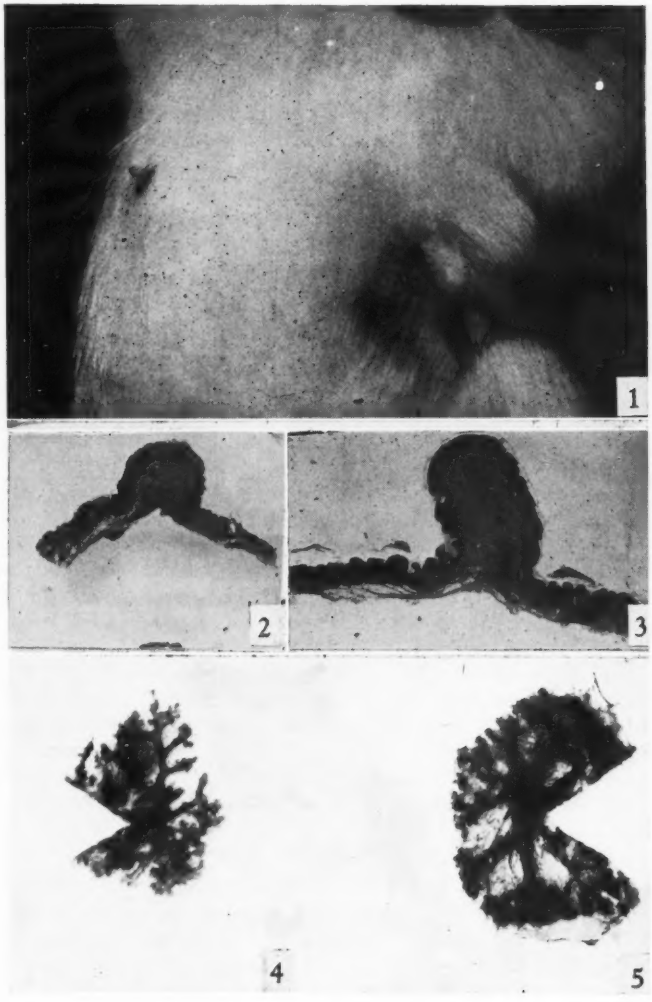

Fig. 1. MAMMARY GLANDS OF A YOUNG MALE MONKEY AFTER UNILATERAL CUTANEOUS TREATMENT WITH ESTROGEN IN ALCOHOL ON LEFT BREAST. Alcohol alone was used as control treatment on the right breast. Fig. 2. SECTION OF THE CONTROL NIPPLE. Low power magnification. Fig. 3. SECTION OF THE LEFT NIPPLE (EXPERIMENTAL) AT THE SAME MAGNIFICATION AS FIGURE 2. Fig. 4. RUDIMENTARY RIGHT MAMMARY GLAND (CONTROL). Fig. 5. LEFT MAMMARY GLAND (EXPERIMENTAL) AT THE SAME MAGNIFICATION AS FIGURE 4.

were shifted, the reddening and swelling of the 'sexual skin' of the right side gradually decreased and was rated 0 in all except one monkey 7 days after the last estrogen treatment. During this time the color of the 'sexual skin' on the left side gradually increased to equal or exceed that attained during the previous period of treatment of the opposite side. A variation in the response of the 'sexual skin' could not be determined within the range of doses used. Color photographs of the 'sexual skin'

were taken after each series of estrone applications to assist in the evaluation of the response.

The left nipples of the two male monkeys which received the local application of estrone on the breast became hyperemic within 4 days after treatment started, and had doubled in size within two weeks. They continued to enlarge for approximately one month (fig. 1). At the same time the untreated nipples increased somewhat in size (table 2) but did not become hyperemic. The skin around the left nipples became irregular and red, forming a pseudoareola (fig. 1).

The mammary glands removed 31 and 126 days after treatment started were slightly larger on the left than on the right side (compare fig. 4 and 5). The variations in size were probably no greater than might be expected in males receiving subcuta-

TABLE 2. LOCAL APPLICATION OF ESTRONE TO THE NIPPLE AND SURROUNDING AREA OF YOUNG MALE MONKEYS (3 μg. daily for 30 and 126 days)

No. of animal	Period treated	Size of nipple at start		Size of nipple at end		Size of glands at end Right	Left
516	36 days	0.5×1.6	2.5×1.6	4.0×3.9	10×5.6	8× 7	10× 8
518	126 days	2.1×1.5	2.1×1.5	3.5×3.5	9×5.4	22×20	22×24

neous injections (12). The rate of development of the glands was not as rapid as following the injection of larger amounts of estrogen. The glands consisted largely of branching ducts terminating in thickened ends. Many mitotic figures were observed in both the glandular parenchyma and stroma of the treated and control breast of *monkey 516*. The epithelium of the ducts was stratified, the cells had not assumed their usual columnar shape.

The nipples of both the right and left breast responded but the changes were much greater on the left sides. Figures 2 and 3 show sections. The thickness of the epithelium as judged by the number of layers of nucleated cells was apparently similar on both sides but the stratum corneum was much thicker on the treated nipples. Much of the hypertrophy of the nipple was due to the increase in cellular and fibrous stroma, vascularity, edema and hypertrophy of the interlacing smooth muscle bundles.

DISCUSSION

The effectiveness of estrogens applied to the skin has been demonstrated previously. Few investigations have been undertaken which attempt to localize the response to the side of application. The 'sexual skin' and the nipples of some primates provide ideal test objects for evidence of the effectiveness of estrogens to local areas. The response of the 'sexual skin' on one side to locally applied estrone, without evidence of response on the opposite side, indicates that estrogen acts directly, and may act in amounts much below that required for systemic effects.

The nipples, like the 'sexual skin' may also respond to the local application of estrogens. The present experiments did not afford critical proof of local stimulation of one mammary gland only, for definite but distinctly less growth occurred on the non-stimulated side. It is probable that the amounts of hormone used were too large. Speert's illustration (13, p. 461) shows more complete localization.

The effectiveness of such small amounts of estrone, 120 to 360 I.U. maximum total dose in 12 days in the females is of special interest. Though it is difficult to interpret international units in terms of rat units because of the great individual fluctuations in the various assays, this is approximately 4 to 12 R.U. total dose in 12 days for the longest treatment of the 'sexual skin'. It requires from 80 to 100 R.U. in 10 days to

stimulate the uterus of the ovariectomized adult to the point where menstruation follows estrogen withdrawal (7).

SUMMARY

Small doses of estrogen in alcohol applied cutaneously induce local responses in the 'sexual skin' of immature female monkeys. Estrone in alcohol (1 to 3γ daily for 8 and 12 days) on one side produced a unilateral reaction while alcohol alone on the other side furnished a control.

Similar treatment (3γ daily for 30 and 12 days) on one breast of young male monkeys induced considerably more growth in both nipple and rudimentary glandular tissue of that side. The other mammary gland showed slight growth.

This type of result encourages use of estrogens locally in small doses, clearly below the threshold for systemic results, in such cases as atrophic diseases of the vulva and asymmetrical development of the breasts.

REFERENCES

1. Zondek, B.: *Lancet* 2: 842. 1936.
2. Moore, C. R., J. K. Lamar and N. Beck: J.A.M.A. 111: 11. 1938.
3. Zondek, B.: *Lancet* 1: 1107. 1938.
4. Deanesly, R., and A. S. Parkes: *Proc. Roy. Soc. London* 124: 279. 1937.
5. Lyons, W. R., and H. S. Templeton: *Proc. Soc. Exper. Biol. & Med.* 33: 587. 1936.
6. Emmens, C. W., and R. J. Ludford: *Nature* 145: 746. 1940.
7. Allen, E.: *Contrib. Embryol. Carnegie Inst. Wash.* 19: 1. 1927.
8. Strong, L. C., W. U. Gardner and R. T. Hill: *Endocrinology* 21: 268. 1937.
9. Gardner, W. U., A. W. Diddle, E. Allen and L. C. Strong: *Anat. Rec.* 60: 457. 1934.
10. Allen, E., F. L. Hisaw and W. U. Gardner: The endocrine functions of the ovaries. Chapt. VIII, Sex and Internal Secretions, E. Allen, C. H. Danforth and E. A. Doisy, Editors. Williams & Wilkins, Baltimore, 1939.
Gardner, W. U.: Influence of estrogenic hormones on abnormal growths. pp. 67, Some Fundamental Aspects of the Cancer Problem, H. B. Ward, Editor. The Science Press, New York. 1937.
11. Gardner, W. U., and T. L. Chamberlin: *Yale J. Biol. & Med.* 1940.
12. Gardner, W. U., and G. van Wagenen: *Endocrinology* 22: 164. 1938.
13. Allen, E., T. H. Burford and W. U. Gardner: *Am. J. Physiol.* 117: 381. 1936.
14. Speert, H.: *Science* 92: 461. 1940.

EFFECT OF ALPHA ESTRADIOL BENZOATE ON LOCAL AREAS OF INFLAMMATION IN THE SKIN OF THE RABBIT[1]

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THERE ARE MANY OBSERVATIONS to show that hormones have a favorable effect upon infections. The mechanism of such action is not well understood. Sprunt (1) has expressed the opinion that all the cells of the body are influenced by pregnancy. This effect results from alterations, in the estrogenic and probably other hormones, associated with pregnancy. The reactivity of the tissues during pregnancy is altered to the virus of infectious myxomatosis. Larger amounts of this virus are required to produce infection in rabbits that are castrated and given estrogenic hormones than are required to produce infection in castrates without the hormone. Pregnant animals are more resistant to vaccinia injection than non-pregnant ones (2). It is well known that pregnancy affects the course of syphilis (3).

Ziskin and associates (4) have observed that the daily injection of follutein following bilateral oöphorectomy produces a clinical picture resembling Vincent's infection. The gums become hard, increase in thickness, and lose all tendency to bleed easily when the estrogenic hormone is given. Follutein probably exerts its influence directly on the gums and oral mucous membranes (5).

Hartman and Scott (6) found that the resistance of adrenal-deficient rats to bacterial intoxication is significantly increased by an extract of the adrenal cortex. This is true for both the acute intoxication produced by killed *B. Typhosus* and for the chronic intoxication produced by killed staphylococci. Campbell (7), by the injection of sex hormones, influenced the normal sexual variations in the resistance of rats to *C. Crassicollis*. He states that "any explanation of such reactions must await further investigation on the physiological and cellular effects of such substances."

Mudd and associates (8) in 1934 reviewed the literature on the effects of hormones on phagocytosis. They found some observations to indicate that certain of the glands of internal secretion influence phagocytosis. A majority of the observations discussed in this review, however, were made in vitro.

Capillary permeability and inflammation and the factors that influence these processes have been discussed (9–13). The present paper is a report of our observations on the effects of alpha estradiol benzoate[2] on capillary permeability and the local inflammatory reaction in the skin of the rabbit.

METHODS AND MATERIALS

The ovaries were removed from 16 adult rabbits with either nembutal or ether as the anesthetic agent. Nine of the animals were kept for 4 weeks, after which 5 were given daily intramuscular injections of 1 cc. (500 R. U.) of alpha estradiol benzoate for 14 days. Four rabbits were kept as controls.

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The skin of these 9 rabbits was carefully shaved several days before the completion of the series of injections of the hormones. The day following the last injection of the hormone xylol was applied with a cotton applicator to local areas of the skin, 24 hours, 4 hours, 2 hours, 30 minutes and 0 minutes, before 10 cc. of a 0.2% solution of trypan blue was given intravenously. The technic used in this experiment is described in detail elsewhere (9). The xylol treated areas of skin were observed for 30 minutes for the appearance of a blue color.

Two-tenths of a cubic centimeter of a heavy saline suspension of washed staphylococci was injected intradermally into 3 different areas in each of the 9 rabbits 6 hours, 4 hours, and 2 hours before the animals were killed. The skin in the areas where the bacteria were injected was removed and placed immediately into a 10% solution of formalin. Paraffin sections were prepared from the skin and stained with hemotoxylin and eosin.

Seven rabbits following oöphorectomy were kept for 2 months at which time 4 were given the same quantity of alpha estradiol benzoate as was given to the above rabbits. Three of the rabbits in this group were kept for controls. The skin of these 7 rabbits was shaved and each animal was injected intradermally with 0.2 cc. of a saline suspension of staphylococci similar to that given to the other rabbits and 0.2 cc. of a 1% solution of aleuronat 6 hours, 4 hours, and 2 hours before they were killed. Xylol was applied to the skin of each of these rabbits at intervals corresponding to that used for the preceding rabbits. The skin where each irritant was placed was removed and fixed in formalin. The sections were prepared by the paraffin technic and stained with hematoxylin and eosin.

The rabbits were weighed before and after the series of injections of the hormone.

EXPERIMENTAL RESULTS

Observations on capillary permeability. Hyperemia occurred in the skin of the rabbits in this experiment within 15 to 60 seconds following the local application of xylol. Edema began very quickly following the development of hyperemia. Both the edema and hyperemia persisted for the duration of the experiment. The time in which the hyperemia and edema developed was the same in the castrates given the hormone as in the controls. There was no difference in the time of appearance and the development of the macroscopic changes in the oöphorectomized rabbits and normal animals similarly treated with xylol and discussed in other experiments (9).

The xylol treated areas of skin in both the experimental and control animals became blue within several minutes following the intravenous injection of trypan blue provided the dye was given immediately following the application of the irritant. There was a progressive diminution in the quantity of dye that localized and concentrated in the xylol treated areas of skin when the interval between the application of the xylol and the injection of the dye was increased. The time and the quantity of dye that localized in the skin of the rabbits in this experiment was the same as observed in normal rabbits similarly treated and reported in other experiments (9).

Observations on the development of the local inflammatory reaction in the skin. The rapidity of the development of the macroscopic changes produced in the skin by staphylococci, aleuronat, and xylol was the same in the rabbits that were castrated and given the hormone as in the controls.

The histological sections showed the presence of polymorphonuclear leucocytes where the staphylococci were injected 2 hours before the animals were killed. The number of leucocytes were increased in those areas where the bacteria were injected 4 and 6 hours previously as compared with the 2-hour lesions. Apparently there was no difference in the number of leucocytes about the bacteria in the oöphorectomized

animals given alpha estradiol benzoate and the controls. There was no difference in the reaction in the skin of the rabbits that were castrated and the normal animals given similar injections of staphylococci.

There was no variation in the histological reaction in the skin, in the experimental and control groups of rabbits where either aleuronat was injected intradermally or xylol was applied locally.

The uterus and fallopian tubes were markedly swollen and hyperemic in all of the rabbits given the hormone. The internal reproductive organs of the rabbits that were castrated appeared to be atrophic. There was no significant difference in the weight of the rabbits given the hormone and the controls.

DISCUSSION

It has been shown and discussed elsewhere that trypan blue localizes and concentrates in areas of inflammation as the result of a change that occurs in the epithelial cell, the connective tissue cells and the endothelial cell of the small blood vessels (13). The present experimental observations indicate that the ovarian hormone, alpha estradiol benzoate, does not produce any noticeable affect on either the action of xylol upon the epithelial cells or the subsequent changes that occur in the connective tissue and the endothelial cells in the local areas of inflammation. This opinion is based upon the localization and the concentration of trypan blue in areas of inflammation following an intravenous injection in the rabbit. The inflammatory reactions as produced by staphylococci, aleuronat and xylol apparently are not influenced either by the absence of the ovaries or the presence of large quantities of alpha estradiol benzoate in the rabbit.

It is conceiveable that certain metabolic processes are influenced by the presence of ovarian hormones. It would not follow necessarily, however, that such a change would affect the rate that a colloid such as trypan blue passes the endothelium of the small blood vessels and become either absorbed or adsorbed by the connective tissue cells and the epithelial cells in areas of inflammation. Likewise, it is conceiveable that the tissue changes associated with the escape of leucocytes from the blood vessels may not be influenced by an ovarian hormone. The results of the present experimental studies indicate that alpha estradiol benzoate has no effect on either the localization and concentration of trypan blue in areas of inflammation produced by the local application of xylol to the skin of the rabbit or upon the escape of polymorphonuclear cells from the vascular system of the rabbits.

The variation in the gross appearance of the uterus and the fallopian tubes in the oöphorectomized animals indicate that the quantity of hormone injected in these experiments is sufficient to produce definite changes in the rabbit.

REFERENCES

1. Sprunt, D. H.: *J. Exper. Med.* 56: 601. 1932.
2. Rosalin, P. D., C. K. Hu and L. Pearce: *J. Immunol.* 31: 59. 1936.
3. Kemp, J. E.: *J. Inf. Dis.* 60: 32. 1937.
4. Ziskin, J. E., S. N. Blackberg and C. A. Slavetz: *J. Dent. Res.* 15–16: 407. 1935–37.
5. Ziskin, D. E.: *J. Dent. Res.* 16: 367. 1937.
6. Hartman, F. A., and W. J. M. Scott: *J. Exper. Med.* 55: 63. 1932.
7. Campbell, D. H.: *Science* 89: 415. 1939.
8. Mudd, S., M. McCutcheon and B. Luche: *Physiol. Rev.* 14: 210. 1934.
9. Rigdon, R. H.: *Arch. Surg.* 41: 101. 1940.
10. Rigdon, R. H.: *Surgery* 8: 839. 1940.
11. Rigdon, R. H.: *Arch. Surg.* 41: 879. 1940.
12. Rigdon, R. H.: *J. Lab. & Clin. Med.* To be published.
13. Rigdon, R. H.: Report to Section of Pathology, Southern Medical Association, Louisville, Ky. November, 1940.

REGRESSIVE CHANGES IN THE REPRODUCTIVE SYSTEM OF MALE RATS INDUCED BY STILBESTROL

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THE MODIFICATION of the reproductive organs in mammals by toxins, proteins and extracts has attracted the attention of numerous investigators for approximately forty years. Beginning with the papers on spermatoxins (1, 2) there followed a period in which grafts and crude extracts were used for the purpose of inducing sterility and changes in the sex glands of experimental animals similar to those found in the freemartins of cattle (3–7). At the present time the crystalline hormones from animal and synthetic sources are capable of producing even greater changes in the reproductive organs (8, 9, 10). The data presented here resulted from a study of the effect of stilbestrol on the reproductive organs of the male rat.

PROCEDURE AND MATERIALS

Sexually mature male rats of Wistar stock, inbred for many generations, ranging in weight from approximately 100 to 400 gm., were used in these experiments. They were fed on Purina Dog Chow and water. Three times a week, for a period of 55 days, one groups of 20 rats was given orally, through a stomach tube inserted without anesthesia, 1.0 mg. of stilbestrol[1] which was prepared by dissolving 25 mg. of crystalline stilbestrol in 0.5 cc. normal NaOH solution and then diluting with distilled water to a concentration of 1.0 mg. per cc. Every 5 days, a second group of 20 males, subdivided into two series of 10 rats each, was given 25γ of stilbestrol in 0.1 cc. sesame oil, the one series receiving the dose by stomach tube, the other series by intramuscular injection, The duration of this experiment was 100 days. The controls were usually litter mates of the stilbestrol treated rats; some of these received sesame oil intramuscularly and those remaining were untreated (*groups C* and *D*, table 1).

RESULTS

In about two weeks, the rats receiving the 1.0 mg. of stilbestrol 3 times a week began to show shrinkage of the scrotum, and the testicles, which soon thereafter became difficult to find, were less firm to the touch. Autopsies performed at the end of the 55-day period of treatment, disclosed the scrotum to be similar to that of a castrated rat (fig. 1), and the testes, seminal vesicles, and prostate were so obviously reduced in size that comparison to normal weights seemed unnecessary (fig. 2, 3). Nevertheless, these organs were weighed and found to be only about one-sixth or less the weights obtained in the normal controls (*groups A* and *B*, table 1). The mean weight of the prostate, which had become so reduced in size that identification was difficult, was only 21 mg. in contrast to 306 mg. in the controls (table 1).

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TABLE 1

Groups	Weights				
	Body	Testes	Epididymides	Seminal vesicles	Prostate
	gm.	mg.	mg.	mg.	mg.
A	178	460	203	77	21
B	200	2465	832	813	306
C	234	2066	664	437	178
D	264	2615	962	876	324
E	2.3	4.3	5.5	3.8	3.4

A, 20 rats receiving 1.0 mg. of stilbestrol in aqueous solution by stomach tube 3 times a week for 55 days.
B, 20 untreated controls.
C, 20 rats given 1/40 mg. stilbestrol in 0.1 cc. sesame oil every 5 days for 100 days, 10 rats by stomach tube and 10 rats by intramuscular injection.
D, 20 control rats, 10 of which received 0.1 cc. sesame oil every 5 days for 100 days.
E, The mean difference of *groups C* and *D* divided by the standard error of the difference.

S.E. of Diff. $=\sqrt{(SE_1)^2+(SE_2)^2}$
S.E. = S.E. of Mean $=\sigma/\sqrt{n}$.

A histological study of the testes, seminal vesicles, and prostate was made. Degeneration was especially marked in the testes of the stilbestrol treated rats. The entire gland was shrunken, the tubules were free from intact spermatozoa, and the thin layer of cells lining the tubular wall also showed lack of interstitial cells, mitotic figures were rare, and vaculation marked (fig. 4). The lumen of the tubules contained broken cells and only fragments of degenerated spermatozoa. Although the seminal vesicles were small and free from fluid content, the cells were not broken and appeared nearly normal. The atrophic condition of the prostate gland was also outstanding in this histological picture. The cells were lower than normal and an increase in fibrous connective tissue was evident.

The results were so striking when doses of 1.0 mg. were given as described that for comparison, a smaller dose seemed pertinent. The dose fixed was 25γ (1/40 mg.) of crystalline stilbestrol, to be given in 0.1 cc. sesame oil every 5 days. Since reports have placed stilbestrol from two and one-half (11) to eighty (12) times as potent as theelin, this dose was probably at least equal to 1000 I.U. of theelin. It was also considered similar to the amount of estrin, reported as about 200 U (13), given off in the mouse at each estrous cycle. This dose and the 5-day interval of time between treatments were chosen, therefore, in order to have these factors similar to those occurring in the normal female. The rats given the 25γ of stilbestrol in sesame oil intramuscularly every 5 days were slightly more affected than those given similar doses by stomach tube. Since the difference between them was nominal, the groups were combined (*group C*, table 1) and compared to the controls (*group D*, table 1). The body weights, although nearly identical at the start, 100 gm. in the treated group and 97 gm. in the controls, differed by 30 gm. at the end of the experiment (table 1). The loss of weight, often as much as 20 gm. during the first 5 days, in animals treated with stilbestrol has been one of the most constant effects noted in these experiments as well as in other reports (14, 15).

The gross appearance of the scrotum showed the skin only slightly more shrunken than normal, and the testes were usually found in the scrotum. The mean weights of the testes, epididymides, seminal vesicles and prostates were all significantly smaller in the stilbestrol treated rats. The mean difference divided by the standard error of the difference was 4.3 for the testes, 5.5 for the epididymides, 3.8 for the seminal vesicles, and 3.4 for the prostate glands (table 1). On microscopic examination

cellular changes were manifest in the testes but many apparently normal sperm were present, and the pathological changes as a whole were slight. In some tubules the spermatids and spermatocytes were desquamated into the lumen; in other tubules the structure was normal.

DISCUSSION

Stilbestrol seems to have a selective action on the testes, affecting it either directly or indirectly more than any other tissues. In these rats the pituitary and adrenal glands were increased in weight but, with the exception of the thymus, no other organ was greatly reduced in size. Data concerning changes in other organs will

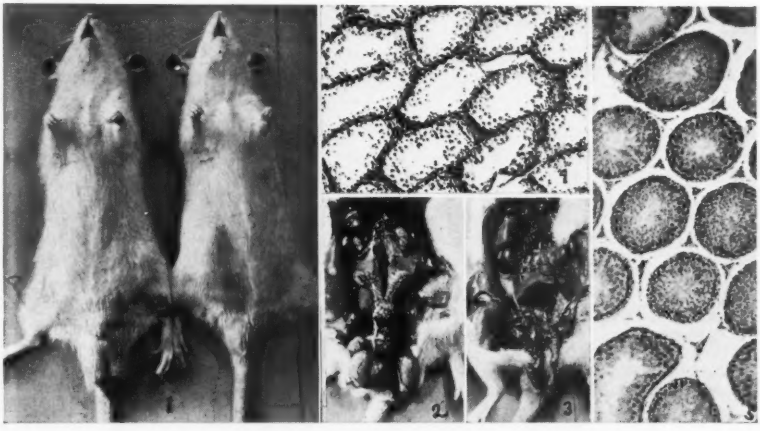

Fig. 1. Normal male rat, body weight 213 gm. (left), compared to a rat of the same age weighing 202 gm. and given 1 mg. of stilbestrol 3 times a week for a period of 55 days. Fig. 2. Reproductive organs of the normal rat, shown in figure 1.

Fig. 3. Reproductive organs of the stilbestrol treated rat, shown in figure 1. Fig. 4. Testis of the stilbestrol treated rat, shown in figure 1 and 3. ×60. Fig. 5. Testis of a normal rat. × 60.

be presented in the future and are merely mentioned here as confirmatory evidence of the specific selective action of stilbestrol for the testes. On the other hand, the gonad retrogression may be secondary to pituitary changes induced by many estrogenic substances (16), including stilbestrol (14, 15) and preliminary experiments in this laboratory. Once the degeneration of the testes has progressed to such an extent that they become located within the abdomen, then the temperature also becomes a factor (17) leading to degeneration. In these experiments all the rats in *group A* (table 1) appeared as though castrated, and the testes could be pressed into the scrotum only by considerable manipulation. It may be concluded therefore that these testes probably were never out of the abdomen during the last few weeks of the experiment. However, when the amount of stilbestrol given was small (*group C* table 1), the testes remained in the scrotum constantly and were not at any time observed to be in the abdomen. Accordingly, the changes found in this group were no doubt due entirely to the smaller dose of stilbestrol. These observations support the finding that some degeneration of the testes can be induced independently of temperature (18).

Sexual desire was lost during treatment with stilbestrol about the time the testes became noticeably shrunken. Copulation was not observed at any time nor would

fertilization have been possible anyway because lack of spermatozoa was one of the effects noted in these rats treated with 1.0 mg. of stilbestrol. Older male animals (one year) noticed females less readily than did the younger males, and the testes of the former were more quickly affected as judged by their appearance and palpation.

SUMMARY

Stilbestrol was given by stomach tube and intramuscular injection to 40 young male rats. The following effects on body weight and the reproductive organs were noted.

About 2 weeks after treatment had been instigated, the scrotum was noticeably shrunken, and the testes were softer than normal. A few weeks later the testes were located in the abdomen, and the scrotum resembled that of a castrated rat.

Interest in female rats in estrus was absent, and copulation was not observed.

At autopsy, the testes, epididymides, prostate and seminal vesicles appeared markedly atrophied. This observation was confirmed by histological examination. Azoospermia and desquamation of the cells lining the tubules were marked.

Body growth was retarded.

REFERENCES

1. Landsteiner, K.: *Centralbl. f. Bakteriologie* 25: 546. 1899.
2. Metchikoff, E.: *Ann. Inst. Pasteur* 14: 24. 1900.
3. Sand, K.: *J. Physiol.* 53: 257. 1919.
4. Moore, C. R.: *J. Exper. Zool.* 33: 365. 1921.
5. McCartney, J. L.: *Am. J. Physiol.* 63: 207. 1923.
6. Emery, F. E.: *Trans. Am. Micro. Soc.* 45: 44. 1926.
7. Pommerenke, W. T.: *Physiol. Zool.* 1: 97. 1928.
8. Hain, A. M.: *Quart. J. Exper. Physiol.* 25: 131. 1935.
9. Turner, C. D., and W. L. Burkhardt: *Proc. Soc. Exper. Biol. & Med.* 42: 267. 1939.
10. Green, R. R. et al.: *Am. J. Anat.* 67: 305. 1940.
11. Dodds, E. C. et al.: *Lancet* 1: 1389. 1938.
12. Sondern, C. W., and J. L. Sealey: *Endocrinology* 27: 670. 1940.
13. Marrian, G. F., and A. S. Parkes: *J. Physiol.* 69: 372. 1930.
14. Noble, R. L.: *Lancet* 2: 192. 1938.
15. Mellish, C. H. et al.: *Endocrinology* 26: 273. 1940.
16. Allen, E. et al.: Sex and Internal Secretions. Williams & Wilkins Co., Baltimore, 1939.
17. Fukui, N.: *Japan Med. World* 3: 160. 1923.
18. Emery, F. E.: *Anat. Rec.* 44: 369. 1930.

VITAMIN A AND THE VAGINAL RESPONSE TO SEX HORMONES IN THE RAT[1]

I. ESTROGENS

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IN 1922 Evans and Bishop (1, 2) reported that vitamin A deficiency causes continuous cornification of the vaginal epithelium in the rat. This condition was found in both intact and castrated animals. In the intact rat, this persistent cornification is independent of the normal estrous cycles which indicates that neither the ovary (directly) nor the hypophysis (indirectly) is involved. The obvious inference is that the vaginal epithelium itself is somehow altered.

In view of the fact that cornification of the castrate rat vagina is used as a criterion for estrogenic activity in biological assay methods, it seemed of value to study the possible relationship between vitamin A intake and the sensitivity of the rat's vaginal epithelium to estrogens. This appeared particularly pertinent since Sherwood, Brend and Roper (3) reported that administration of excess vitamin A to normal female rats suppressed the normal vaginal cornification of estrus.

A group of 36 mature castrate females which had been used for an estrogen assay were divided into 2 groups. One group was given a normal diet of rat chows plus excess vitamin A; the other group was placed on a specially prepared diet containing no vitamin A.[2] The excess vitamin A[3] was fed orally by means of a tuberculin syringe. Each rat received 5000 U.S.P. U daily in addition to the quantity in the normal diet. At approximately weekly intervals both groups of rats were tested for their response to 1.0γ of estrone with an occasional priming dose of 5.0γ to maintain sensitivity.

Theoretically the animals on the vitamin A free diet should have exhibited an increase in percentage response to the estrogen or should have responded to even smaller doses of estrogen as their vitamin A reserves were depleted. This was not the case. The percentage response of these animals fluctuated from week to week as it had before the dietary regime was begun. Compared to the pre-experimental period there was no evidence of increased sensitivity during the entire observation period of 9 months.

It should be explained that the mature rat has a considerable vitamin A reserve which is depleted only after months on an A free diet. Toward the end of the experimental period, the animals began to show signs of A deficiency such as the presence of considerable numbers of cornified cells in the vaginal smears when no estrogens were administered. This condition, however, was not confused with that of a true

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[2] The diet used was made up according to the directions in Practical Physiological Chemistry by Hawk and Bergheim (1937), p. 886. Chemical tests of the ingredients and a biological test of the diet showed it to be lacking in vitamin A.

[3] Abbot-Fish liver oil concentrate—300,000 U. vitamin A per gm. and 2,850 U vitamin D—diluted with peanut oil to a concentration of 5,000 U per 0.2 cc. (daily dose administered).

response to the administered estrogen since a vaginal smear was considered positive only when leucocytes were completely absent and cornified and nucleated epithelial cells were present in large quantities.

The animals receiving the excess vitamin A were expected to show a loss of sensitivity to estrogens in view of the reported finding that the vaginal epithelium of normal animals fails to cornify under such treatment. After 4 months of continued administration of vitamin A these animals still retained their capacity to respond to estrogenic stimulation by vaginal cornification.

This failure to find any relationship between the vaginal response to estrogens and the vitamin A content of the body prompted us to check the reported effect of excess vitamin A on the vagina of the normal rat. The effect of A deficiency on the vaginal epithelium has been well established by observations from many laboratories. The inhibitory effect of excess vitamin A on vaginal cornification, to our knowledge, has been reported by only one group of investigators.

Smears were taken daily for 15 days on 12 females, 150 ± 10 gm. in weight, to determine the normalcy of their estrous cycles. One animal with abnormal cycles was discarded. Beginning on the 15th day, each animal was given 5,000 U.S.P. U of vitamin A daily in addition to the standard diet of rat chows. The vitamin was administered in the manner already described. The daily treatment was continued for 30 days during which time daily smears were made. In all but two of the 11 animals normal cycles with rhythmic occurrence of full estrous smears continued for the 30-day treatment period. Only slight variations in the length of the cycle and duration of the estrous phase were found and these were no more marked than those which had also occurred before treatment was begun. In the two exceptions, prolonged periods of diestrus, 12 and 13 days respectively, occurred. Since regular cycles were resumed in both cases after these periods, it seemed probable that they represented conditions of pseudopregnancy probably caused by accidental stimulation of the cervix during the smearing procedure. The vaginal smears obtained during the treatment period showed no differences from those of the control period. During estrus they were composed of the usual large numbers of typical cornified epithelial cells.

In contrast to this lack of effect in our animals was the very marked change observed by Sherwood, Brend and Roper (3). Two days after the onset of treatment with vitamin A they found changes in the character of the smears and, during the 15 days of treatment, the smears never progressed from the nucleated epithelial stage to the cornified stage. Large numbers of nucleated epithelial cells and leucocytes were found continuously, but only occasionally were a few cornified cells present. These conditions they found to persist even 20 days after cessation of treatment.

The two procedures differed only in the substances used and the amounts involved. Sherwood, Brend and Roper used 1,500 to 1,375 I.U. carotene (provitamin A) whereas we used pure vitamin A in doses of 5,000 I.U. (U.S.P. units). No explanation of the discrepancy in the results of the two studies can be offered.

SUMMARY

Vitamin A deficiency does not increase the sensitivity of the castrate rat vagina to administered estrogens. Excess vitamin A does not inhibit the response of the vagina to administered estrogens in the castrate nor the response to endogenous estrogens in the normal rat.

REFERENCES

1. Evans, H. M., and K. S. Bishop: *J. Metab. Res.* 1: 335. 1922.
2. Evans, H. M., and K. S. Bishop: *Anat. Rec.* 23: 17. 1922.
3. Sherwood, T. C., M. A. Brend and E. A. Roper: *J. Nutrition* 11: 593. 1936.

VITAMIN A AND THE VAGINAL RESPONSE TO SEX HORMONES IN THE RAT[1]

II. ANDROGENS

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IN 1933, Aberle (1) reported that the vagina of the A deficient rat fails to respond to a placental extract which, in the normal rat, indirectly produces mucification of the vaginal epithelium. The absence of mucification in the vaginal epithelium of the A deficient pregnant rat has also been reported (2). The present report concerns the response of the vagina of the A deficient castrate rat to testosterone propionate.

A group of 9 female rats, 42 to 45 days old, were placed on an A deficient diet.[2] After one month on this diet, a fair proportion of squamous epithelial cells began to appear and persisted in the daily vaginal smears. The animals were therefore considered to be A deficient and were castrated. One week after castration 6 of these rats were given different doses of testosterone propionate[3] (0.05 to 5.0 mg.). No marked changes occurred in the smears up to 96 hours after administration. At 96 hours the animal receiving the highest dose of testosterone propionate (5.0 mg.) was killed. Microscopic examination of the vaginal epithelium showed no cornification and a typical picture of early mucification. The epithelium was several layers thick with a superficial layer of high columnar cells. A few areas of mucoid degeneration were also present.

The 8 remaining animals were maintained on the A free diet and 8 weeks later vaginal smears were made. These smears were stained with gram stain which specifically stains keratin blue. Surprisingly, the smears showed no true keratinized cells although in the fresh smears considerable quantities of squamous epithelial cells were present. Five weeks later (after 4+ months on the diet) the gram stained smears showed numerous keratinized cells in all but one animal. Whether or not a condition of desquamation without keratinization should be considered an indication of A deficiency cannot be considered here. It is apparent, however, that increased mitotic activity and superficial sloughing of squamous cells does occur at an earlier period of A depletion than true keratinization.

One week after the last smearing the animals were given testosterone propionate subcutaneously, 4.0 mg. daily for 6 days. On the 7th day the animals were killed and sections from the middle region of the vagina were prepared for histological study.

With one exception the vaginas of these animals were cornified and showed no evidence of mucification. The one animal which did not have a cornified vagina

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[2] Same diet used in previous study (3).

[3] The testosterone propionate (Perandren) was kindly furnished by Ciba Pharmaceutical Products, Inc.

showed a typical mucification response to the testosterone propionate. As in the first animal tested, earlier in the experiment, superficial high columnar cells were found together with some areas of mucoid degeneration. This animal was the same one which had only a few keratinized cells in the gram stained smear. It seems probable that this animal was not so completely A depleted as the remaining animals which all had numerous keratinized cells in the vaginal smears. The vagina of this animal and the animal tested earlier in the experiment responded to testosterone propionate by characteristic mucification whereas the rest failed to respond.

The urethras from 6 of the animals were also examined histologically. Three showed marked metaplasia with superficial cornification of the urethral epithelium. Two of these latter animals had shown conditions of xeropthalmia indicative of advanced A deficiency.

In order to test the adequacy of the dose of testosterone propionate used in the previous procedure 3 old castrate females were given various doses as follows: 4 mg. daily for 6 days; 2 mg. daily for 6 days and 2 mg. daily for 10 days. In all 3 the vagina showed a typical mucification.

As a further check to rule out the possibility of insufficient dosage, 3 more animals were used. These animals had been placed on the A free diet soon after weaning. After 1+ months on the diet these animals exhibited the usual symptoms of A deficiency and were castrated. Two were given 8 mg. of testosterone propionate daily for 6 days and were killed on the 7th day. The third was given no treatment and served as a control. The vaginas of all these animals, when microscopically examined, showed stratification and surface cornification of the epithelium with considerable leucocytic infiltration. No evidence of mucification was found in the two which had each received 48 mg. of testosterone propionate, twice the amount given to larger animals in the earlier procedure.

SUMMARY

Vitamin A deficiency accompanied by cornification of the vaginal epithelium inhibits the mucifying action of testosterone propionate on the epithelium in the castrate rat.

REFERENCES

1. Aberle, S. B. D.: *Am. J. Physiol.* 106: 267. 1933.
2. Mason, K. E., and E. T. Ellison. *J. Nutrition* 9: 735. 1935.
3. Burrill, M. W., and R. R. Greene: *Endocrinology.* 28: 765. 1941.

ENDOCRINE FACTORS INFLUENCING TUMOR DEVELOPMENT[1]

THE EFFECT OF THE GONADOTROPINS AND OF THEELIN UPON THE MARSH-BUFFALO ADENOCARCINOMA AND LYMPHOSARCOMA

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IT IS GENERALLY CONCEDED that the mammary gland adenocarcinoma which occurs spontaneously in a number of strains of mice can occur only in a gland which has undergone a certain degree of development (1). This development is dependent upon the hormones of the ovary and pituitary and possibly adrenals. The explanation of the mechanism is still controversial, there being two theories: *a*), that the hormones or their products of metabolism have a direct carcinogenic effect; or *b*), that the hormones merely produce the anatomical development of the gland and in so doing give the opportunity for a mutation in the multiplying cells, leading to the production of carcinoma. We previously pointed out that in experiments recorded so far the estrogenic hormones used to promote enhanced or early cancer formation have always been of exogenous origin (2). In order to study the effect of endogenous estrin-like substances, prolan was administered parenterally so as to stimulate the ovaries of mice of the Marsh-Buffalo strain to excessive hormone production. This treatment produced a marked hyperplasia of the ovaries and presumably a marked hyperovarian function, but it failed to influence the onset or incidence of adenocarcinoma of the breast to which this strain is subject and which is normally enhanced in breeding animals.

The present report is a continuation of these studies which led to certain obvious deductions. Since the prolan was shown to produce acinar development in the mammary gland, it was evident that this particular hyperplasia did not lead to enhanced cell mutation. Either prolan stimulated the ovaries to the production of estrogenic substances which did not enhance carcinogenesis or the amounts of estrogenic substances produced were not liberated in sufficient amounts or were destroyed by some defense mechanism.

In this paper are recorded the following experiments: *a*), determination of the minimum dose of exogenous theelin which produces enhanced carcinogenesis; *b*), the influence of prolan given before sexual development; *c*), the influence of massive doses of prolan; *d*), the influence of pregnant mare's serum; *e*), the influence of the pituitary gonadotropic hormone administered to produce maximum ovarian stimulation; *f*), the influence of the pituitary gonadotropic hormone administered to produce the antagonist effect. Histologic studies of the ovaries and mammary glands were made during the course of the study in order to correlate if possible any changes produced with tumor development or inhibition.

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Virgin mice of the Marsh-Buffalo strain served as the experimental animal. Not less than 30 and usually 40 to 50 mice with equal numbers of litter mates as controls were used per experiment. Data based on fewer numbers of mice per experiment and the failure to institute litter-mate controls have introduced a state of confusion (3) in cancer experimentation.

Theelin

Theelin in females (800 Doisy units on alternate months). Three sets of litter mates, comprising 30 mice in a set, were divided into one control and two theelin-dosed series. The one-dosed series received weekly doses of 0.066 mg. theelin in oil (200 Doisy U per week). The other series received the same dosage per week for a 4-week period with an alternate period without dosage. The material first injected (the first 5 months of the experiment) contained 0.2 mg. theelin per cc. oil. Later a more concentrated mixture, 1.0 mg. theelin per cc. oil was used. Dosage was terminated the 11th month of life, the first dose having been administered at the age of 30 days. In the series receiving continuous theelin dosage, 43% had succumbed at the age of 9 months and 90% at the age of 12 months. Only 1 adenocarcinoma of the breast appeared and that at 9 months. Death followed pneumonia in almost every case. This series, therefore, gave no information as to the carcinogenic effect of theelin. In the series receiving theelin injections on alternate months, only 1 mouse died during the period of dosage of causes other than those caused by tumor development. Each mouse received in all 1.4 mg. theelin. The data for the completed experiment showed 80% of the mice developed adenocarcinoma of the breast, 10% developed lymphosarcoma, and 10% died of other causes. For the corresponding controls, 77% developed adenocarcinoma of the breast, 7% developed lymphosarcoma, 3% developed fibrosarcoma, and 13% died of causes not related to tumor formation.

Theelin in females (2100 Doisy units per month). Forty pairs of litter mates 40 to 70 days of age were studied. The controls received 2 weekly doses of 0.04 cc. sesame oil per mouse intramuscularly. The theelin treated mice received the same amount of oil containing 2 mg. theelin per cc. After 5 months of dosage, 10 theelin treated mice and 4 control mice had succumbed, death usually following dosage. The dose was then cut in half. A month later dosage was terminated as 2 more of the theelin treated mice succumbed. Tumor incidence has been calculated for 30 theelin treated and 35 control mice, those mice succumbing before the 8th month of age being eliminated from the experiment. A comparision of litter mates was not made, statistics being based on random sampling. In this experiment, each of the theelin treated mice received 3.8 mg. crystalline theelin in 2 cc. sesame oil in 6 months. The controls received the same amount of sesame oil. The experiment was terminated when the mice had reached the age of 17 months. At this time 52% of the controls developed adenocarcinoma, 20% developed lymphosarcoma, 5% had died of other causes, and 23% were living. Of the dosed mice 53% developed adenocarcinoma, 36% developed lymphosarcoma, 7% had died of other causes, and 4% were living.

Theelin treated males. Thirty male mice of the Marsh-Buffalo strain each received 100 U (Doisy) theelin in water per week twice daily for 4 months after which the same amount was given in oil by weekly injection for a period of 9 months. Each mouse, therefore, received 1.7 mg. (5200 Doisy U) of theelin. No mammary tumors appeared. The experiment was terminated when the mice reached the age of 20 months. During this period 9 mice developed lymphosarcoma, 5 developed fibrosarcoma, and 2 developed fibroma.

Influence of theelin on mammary glands. Whole mounts were prepared of the breasts of 2-month-old mice of the Marsh-Buffalo strain, which had received 2000

Doisy U per mouse of theelin in 10 days. There was no perceptible difference in the appearance of the breasts of the treated and control males (4 mice). In the females there was a slight but perceptible difference in the appearance of the breasts of the treated and control mice (4 mice). While the maximum diameter of the ducts was no greater in the treated group, their mean diameter was greater; the ducts were longer and budding was just beginning. In another experiment, 1000 Doisy U of amniotin (Squibb) per mouse was administered in 15 days. Histologic examination of the breasts of 5 mice so treated revealed no significant difference in appearance from that of 5 respective controls.

Results of theelin experiments. In the experiment in which 1.4 mg. theelin was administered to female mice during a 10-month period there was no significant difference between the control and treated mice in either the appearance of adenocarcinoma or lymphosarcoma. At the age of 10 months, 7% of the controls and 20% of the treated mice had developed adenocarcinoma. The difference of 13% which was the maximum difference encountered is only 1.5 times the standard deviation of the mean. In the experiment in which 3.8 mg. theelin was administered to female mice during a 5-month period, there is an apparent increase in the number of both adenocarcinoma and lymphosarcoma appearing in the treated mice at an early period. This difference corresponds to 2.4 times the standard deviation of the mean the 9th, 11th, and 12th month for the adenocarcinoma and 2.0 times the standard deviation of the mean the 13th month for lymphosarcoma. At the age of 11 months, 17% of the controls and 43% of the treated mice had developed adenocarcinoma. The 13th month of age, 14% of the controls had developed lymphosarcoma; 36% of the treated mice had developed lymphosarcoma. These figures are doubtfully significant by statistical analysis. The male mice which received 1.7 mg. theelin in 13 months failed to develop breast tumors.

It has been demonstrated that theelin, testosterone, and the extracts of the pituitaries of pregnant cattle produce mammary gland development in male mice. According to Lewis et al. (4), 80 to 200 R.U. of theelin administered in 10 days produced positive results. Testosterone propionate is only effective after 25 days treatment. A total of 105 mg. of pituitary tissue given in 6 days is required for positive results. Prolan gave negative results, when 560 R.U. were administered in 14 days.

A total of 100 R.U. per week of theelin was the dose found by Lacassagne and by Suntzeff, Burns, Moskop and Loeb (5) to produce mammary gland tumors in both males and females in cancer susceptible strains. The dose equals the amount found by Lewis et al. required to produce mammary gland development in male mice. We have previously shown (6) that the males of the Marsh-Buffalo strain are responsive to theelin administration in that atrophy of the gonads and seminal vesicles occurs. The conclusion to be reached is that the Marsh-Buffalo strain, while highly susceptible to development of cancer of the breast, is markedly resistant to the influence of theelin thereon.

Prolan

Dosage before sexual maturity. The final data for tumor incidence are compiled for 42 control and 42 treated mice. Mice used for section and breeding were eliminated from the tumor incidence compilation. In the treated series, each mouse received 60 U of prolan (follutein, Squibb) per month for 12 months. This was administered 15 U per day for 4 consecutive days. The first dose was received at the age of 21 to 23 days. The average age at which vaginal canalization had taken place in the treated mice was 26.7 days, while in the control mice it was 49.8 days. Three pairs of litter mates were killed at the age of 1, 3 and 5 months, the treated mice having received 1, 3 and 5 series

of doses respectively. In each case the ovary of the treated mouse was more heavily luteinized and larger (90 to 450%) than the ovary of the control mouse. At 5 months of age the control mammary gland showed scattered ducts with no budding; the gland of the treated mouse showed budding.

Two of the treated mice were mated at the ages of 13 and 14 months respectively. No young were delivered but dead fetuses were found in the uterus. There was no significant difference in the date of appearance of the adenocarcinoma of the mammary glands in the treated and control mice. The experiment was terminated when the mice had reached the age of 20 months. Four tumors in the treated series and 6 in the control series were lymphosarcomas. A fibrosarcoma of the liver appeared in the control series. One of the mice with a fibrosarcoma also had an adenocarcinoma.

Massive prolan dosage. In this experiment, the mice received 300 U of prolan per mouse each month during a 15-day period. Each treated mouse received in all 2700 U of prolan. The experiment was begun at the age of 60 days. One mouse bred at the age of 9 months became pregnant. The final data are calculated for 33 control and 33 treated mice. The results for the incidence of breast cancer are given in the chart and show that the enormous doses of prolan failed completely to influence carcinogenesis. Fifteen per cent of the treated mice and 21% of the control mice developed lymphosarcoma. Six per cent of the treated mice and 9% of the control mice died of other causes. Dosage of 300 U of prolan per mouse in 15 days produces marked acinar development in the mammary tissue.

Prolan treated males. Sixty-four 1 to 2-month-old males of the Marsh-Buffalo strain were separated into a treated and control colony by random sampling. The treated mice received 40 U prolan per mouse the 1st month, then 60 U per month for 5 months, and finally 120 U per month for 5 months. No mouse developed a mammary tumor. At the age of 20 months, 4 controls and 6 treated mice had developed lymphosarcoma. Three control mice developed fibrosarcoma. One spindle cell sarcoma and 1 fibroma appeared in the treated mice.

In a series of 5 virgin 24 gm. female mice, 15 mg. testosterone propionate per mouse administered in 15 days failed to alter the histologic appearance of the mammary gland as compared with respective litter-mate control material.

Results of prolan experiments. While producing marked acinar development in normal female mice, prolan failed to influence the onset or incidence of the adenocarcinoma of the breast, or lymphosarscoma, whether first administered before sexual maturity (720 U per mouse in 12 months), whether first treated at the age of 60 to 90 days (660 U per mouse in 11 months), or whether given in massive doses (2700 U in 9 months). Male mice which received 940 U prolan in 11 months failed to develop mammary tumors.

Pregnant Mare's Serum

Mare's serum. During the course of the experiment, groups of litter mates were killed for histologic studies. The treated mice received 9 courses of doses in 1 year, beginning at the age of 30 days. The first 3 courses consisted of 50 U each per mouse administered in 10 days. The last 6 courses consisted of 100 U each per mouse administered in 10 days. Each mouse, therefore, received a total of 750 U. Tumor incidence is computed for 38 control and 39 treated mice.

Cutter's pregnant mare's serum assaying 50 U per cc. was used. Tested in rats for estrin by the vaginal smear method of Doisy, 1 cc. gave a positive response; 0.5 cc. gave a negative response.

There was a 5 months lag before carcinogenesis resulted in the treated mice. It was during the early stage of dosage that the effects upon the ovary and mammary

glands were most pronounced (table 1). Thirteen per cent of the controls and 28% of the treated mice developed lymphosarcoma.

A single series of doses of pregnant mare's serum produced effects upon the ovaries and mammary glands of the immature mice. The ovaries of the controls showed follicles in all states of development. The ovaries of the treated mice increased 300% in weight and were heavily luteinized. Whereas the mammary gland of the controls showed only ducts, budding was noted in the treated mice. At 4 months of age no acinar development was noted in the controls; marked development was noted in the treated mice. A similar picture presented itself at the age of 7 months. At the age of

TABLE 1. INFLUENCE OF PREGNANT MARE SERUM UPON OVARIES AND MAMMARY GLAND OF MARSH-BUFFALO MICE

No. of mice	Age of mice	Total dose mare serum received per mouse	Body wt., av.	Ovarian wt., av.	Histology of ovaries	Histology of mammary glands
	days	U	*gm.*	*mg.*		
4	35–45		15.0	3.5	F., no C. L.	Only ducts
4	35–45	50	14.8	12.8	F., C. L.	Early ductal hyperplasia and acinar development
2	90–120		23.0	11.0	F., C. L.	Only ducts
2	90–120	100	23.0	20.5	F., C. L.	Frequent budding, some acinar development
1	210					Ductal development
1	210	310				Marked acinar hyperplasia
3	330–360		25.0	13.0	Few F., C. L.	Acinar development
3	330–360	750	25.0	17.0	Few F., C. L.	Acinar development

F., Follicles
C. L., Corpora lutea

11 months, the ovaries of the treated mice were somewhat larger than those of the controls. Histologically there was no difference at this time between the ovaries and mammary glands of control and treated mice (table 1). Two mice which had received the full course of mare's serum dosage were mated at 1 year of age and became pregnant.

Purified mare's serum hormone. Forty-six pairs of litter-mate female mice were originally used for this experiment. Dosage was begun at approximately 30 days of age. The dosed series received 5 U of hormone per mouse per day for 10 days. Thirty days later and every succeeding 30 days the series of doses was repeated. At the age of 7 months the dose was doubled. Dosage was continued to the age of 15 months. Each mouse received a total of 950 U mare's serum hormone. The hormone was prepared by Cutter Laboratories in a high degree of purity, each 200 U being contained per 0.09 mg. nitrogen.

Unfortunately an epidemic of pneumonia destroyed a large number of the control and dosed mice. Twenty-eight per cent of the controls had died of pneumonia by the 13th month. In presenting the data, some little difficulty has arisen in the compilation because of this disturbing factor. All data of mice litter mates, one of which developed pneumonia before the 14th month of age, have been eliminated from the compilation. No attempt was made to compile data beyond the 13th month period as deaths from pneumonia continued up to the 17th month. The data recorded for the chart are based upon 26 pairs of litter mates. In these 26 pairs, no controls and 1 dosed mouse developed lymphosarcoma.

In a series of 15 daily doses, a total of 150 u hormone per mouse, produced marked acinar development of the mammary gland in 24 gm. female virgin mice whether crude mare's serum or purified hormone was given (15 mice, 3 sets of litter mates).

Results of mare's serum experiments. In both experiments, whether the crude mare's serum or whether the purified hormone was given, the appearance of the adenocarcinoma of the breast was delayed in the animals which received the hormone. At the 12th month of age in the crude hormone experiment, 8% of the dosed mice and 36% of the controls had developed breast tumors; at the age of 17 months 34% of the dosed mice and 67% of the controls had developed tumors. In the second experiment (purified hormone), 11% of the dosed mice and 44% of the controls had developed tumors the 12th month of age.

Pituitary Gonadotropic Extract

In the case of prolan and mare's serum hormone, the route of injection is without influence upon the nature of the ovarian response. In the case of the pituitary gonadotropic hormone, the route of injection is of the greatest importance. The follicular response is practically absent when the intraperitoneal route and 1 injection per day are used, while the luteal response is as extensive when given intraperitoneally as when given subcutaneously. Experiments in this laboratory employing divided dosage (7) have shown that the difference in response is brought about by difference of resorption of the hormone. In studying the influence of the pituitary hormone upon mammary gland development the experiments were designed to answer the question as to whether the luteal phase in comparison to the follicular phase would influence, through hormone stimulation, the mammary gland development to the formation of neoplasms.

Fifty pairs of litter mates were employed in an experiment in which subcutaneous injections of the pituitary hormone were made. Forty-five pairs of litter mates were employed in an experiment in which intraperitoneal injections of the pituitary hormone were made. Injections were begun at the age of 22 to 23 days, the dosed colony receiving 0.4 mg. pituitary powder per mouse daily for a period of 10 days. This procedure was repeated every 30 days during the ensuing 13 months. The same dose and and same material were given in each series, but the subcutaneous injection contained 0.1 mg. Zn per dose, added to the solution, which was then adjusted to pH 8.5. This procedure delays resorption over 12 hours (7, 8). The administration to immature mice of 1.3 mg. of this hormone in Zn combination produced 100% ovarian hypertrophy in 4 days. The preparation used was the aqueous bicarbonate soluble, 80% ethanol insoluble fraction prepared from acetone defatted sheep pituitary glands.

At the age of 15 months in the case of the mice which had received the subcutaneous injections and at the age of 14 months in the case of the mice which had received the intraperitoneal injections, 4 treated mice and 4 control litter mates were killed. The ovaries of the controls of each series averaged 8 mg. in weight. The ovaries of the mice which had received the subcutaneous injections averaged 3.4 mg., those in the intraperitoneal series 2.6 mg. The control ovaries at this age are characterized by extensive luteinization with few follicles. The ovaries of the treated mice also showed luteinization, but the interstitial tissue was more prominent with few follicles. Whole mammary gland mounts showed no significant difference between controls and treated animals, there being, however, considerable normal variation in ductal and acinar development.

Eleven mice which had received the subcutaneous injections were bred at the age of 12 to 16 months. In 9 instances this was done following the discovery of a tumor.

In only 3 mice were fetuses found. Two of the control mice became pregnant and delivered young at the age of 12 to 14 months.

Effect of pituitary powder on mammary gland. In a series of 2 groups of litter mates, 24 gm. female virgin mice, the influence of the pituitary powder was tested upon mammary gland development. Each mouse received 6 mg. powder and 1.5 mg. Zn (PH 8.5) in 15 days. The average ovarian weight of 5 controls was 11.2 mg. The average ovarian weight of the treated mice was 13.2 mg. The mammary glands of the treated mice all showed more acinar development than those of the controls.

Results of pituitary hormone experiments. There is a highly significant influence of the subcutaneous injection of the pituitary hormone in retarding mammary gland carcinogenesis. Up to the period at which dosage was discontinued 65% of the controls and only 20% of the treated mice developed adenocarcinoma of the breast. This difference is five times the standard deviation of the mean. The results of the experiments in which intraperitoneal injections were made are not as striking as those obtained for the series in which subcutaneous injections were made, but the results may be considered significant. In both series, the treated mice showed a higher incidence of lymphosarcoma. For the series receiving subcutaneous injections 10% of the controls, 30% of the dosed mice developed lymphosarcoma. For the series receiving intraperitoneal injections, 4% of the controls and 22% of the treated mice developed lymphosarcoma. The significance of this finding is discussed elsewhere.

Lymphosarcoma

In both control males and females the incidence of lymphosarcoma for the age groups studied ranges between 10 to 20%. The lymphosarcoma is, therefore, not linked to the ovaries as is the adenocarcinoma of the breast. In none of the experiments in which the incidence of adenocarcinoma was not affected by hormone administration was the incidence of lymphosarcoma affected. However, in the pregnant mare's serum experiment, the large dose theelin experiment, and the two pituitary hormone experiments, the treated mice had an increase in lymphosarcoma incidence by 15 to 20%. The increase in lymphosarcoma in those experiments in which there was a decrease in adenocarcinoma is probably due to the fact that by suppression of the appearance of the adenocarcinoma a larger number of mice survived and were available for development of lymphosarcoma. This explanation does not hold for the theelin experiment in which both the incidence of adenocarcinoma and lymphosarcoma is increased. In other strains of mice exogenous theelin increases the incidence of lymphosarcoma and the Marsh-Buffalo strain is apparently no exception (9).

DISCUSSION

Although vaginal canalization takes place at 50 days of age, acinar development in the mammary gland may be found absent at 7 months of age in virgins of the Marsh-Buffalo strain. At the age of a year or later it is present. All three gonadotropic hormones (prolan, pregnant mare's serum, the pituitary) produce acinar development of the mammary gland tissue in young virgin mice, comparable to the development which is normally attained at the age of 1 year or later. However, in those experiments in which histologic studies were made (mare's serum and pituitary hormone) the hormones acting through a period of approximately a year did not at the age of a year or later produce glandular development to a degree greater than was found in their respective untreated litter-mate controls. Theelin does not produce the early acinar development in a comparable period even in sublethal doses. This development, induced by the gonadotropic hormones, is mediated through the ovaries for it does

not occur in castrated animals. Since the gonadotropic hormones producing this development did not enhance carcinogenesis, two of them actually delaying it, we are forced to conclude that carcinogenesis in the Marsh-Buffalo strain is not due to the stimulation required to produce the normal development of the acinar structure to the stage present in adult females, nor does the early development by the gonadotropic hormones lead to the mutation responsible for tumor development. It must be concluded that the mutation to the cancerous process is involved in breast stimulation beyond the normal adult resting state, or in a stimulation deviating from the normal state (ductal hyperplasia).

In the Marsh-Buffalo strain, theelin enhances carcinogenesis only in sublethal doses. It is interesting to note that the enhanced production of endogenous theelin-like substances by all three gonadotropic hormones is not conducive to earlier carcino-

TABLE 2. RELATIVE EFFICACY OF PROLAN AND THEELIN IN PRODUCING VAGINAL CANALIZATION AND UTERINE STIMULATION

Substance	Total dose per mouse	Vaginal canalization	Uterine hypertrophy over litter-mate controls
	I.U.	%	%
Prolan	0.5	100	
	0.2	100	
	0.1	100	350
	0.05	75	50
	0.02	0	0
Theelin	1.5	100	50
	0.6	0	0
	0.3	0	0

Tested upon 22 to 23-day-old mice in 4 doses in 4 days. The results are on groups of 4 or 5 litter mates. The prolan used was Squibb's follutein in their clinical units. Parke, Davis' aqueous crystalline theelin was used.

genesis. Since exogenous theelin does not produce the mammary gland development produced by the secretion of the ovaries acting through gonadotropin stimulation, it is apparent that some other substance is secreted by the ovaries which produces this development. This may or may not be an estrogen. In summing up the work on mammary gland development Turner (10) points out that estrogen will grow the mammary ducts in most species, whereas a combination of estrogen and progestin is required to cause complete lobule-alveolar proliferation. The action is not direct but mediated through the pituitary. It is of course well known that the ovaries produce theelin-like substances through gonadotropin stimulation. The relation between the amount of prolan (exogenous) and the amount of theelin (exogenous) required to produce vaginal canalization is given in table 2. One unit of prolan produces the effect of 15 I.U. of theelin.

In seeking for a clue, we are confronted with the paradox that prolan is without influence, while the mare's serum and pituitary hormones decrease carcinogeneis. In the doses given all three hormones produced heavy luteinization of the ovary. In the case of the mare's serum and prolan, ovarian weights were larger in treated animals after repeated periods of dosage. The pituitary hormone acting intermittently over a year produced smaller ovaries at the age of 15 months. However, there is no histologic evidence that these hormones by continued dosage produced atrophy of the mammary gland. In all cases certain animals were able to become pregnant after a year of dosage.

Loeb and Kirtz (11) showed that transplants of pituitaries from brother or sister mice would survive and lead to increased development of the mammary gland in both

cancer resistant and cancer susceptible strains of mice and to an increased incidence of cancer in the susceptible strains. The fact that injection of pituitary hormones at first led to increased breast development, which effect later was not sustained and that the

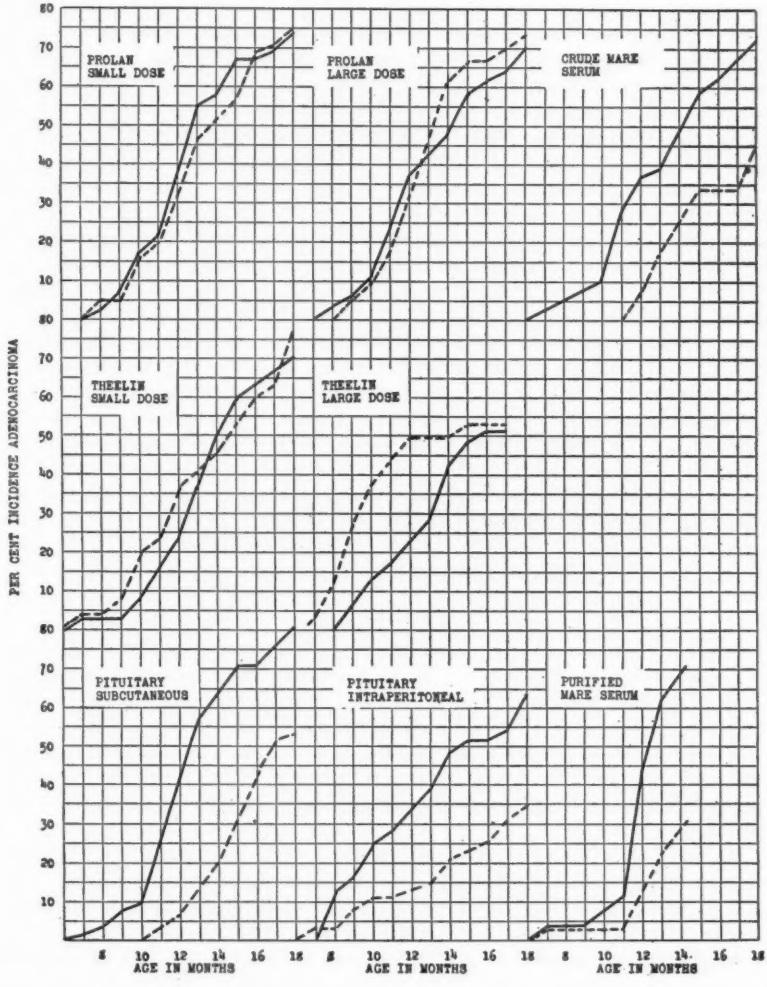

Fig. 1. Influence of prolan, pregnant mare serum, pituitary gonadotropic hormone and theelin upon the incidence of adenocarcinoma of the breast in the Marsh-Buffalo virgin female mouse. The unbroken line represents the development of mammary gland tumors in the control series. The broken line represents the development of mammary gland tumors in the treated series.

cancer incidence was markedly decreased suggests the production of anti-hormone-like bodies by the long continued dosage of these substances in our experiments. Such an anti-effect is apparently not elicited by transplants.

Cramer and Horning (12) have shown that dosage of thyrotropic hormone into

cancer susceptible mice beginning at the age of two months prevented the development of cancer of the breast. Commercial preparations were used and were known to contain some gonadotropic hormone. Since Jensen and Tolksdorf (13) were unable to divorce the thyrotropic factor from the gonadotropic factor in pituitary extracts, it is very possible that our results with pituitary extract are essentially a confirmation of those of Cramer and Horning. Since, however, the mare's serum hormone is without thyrotropic effect and since in our own pituitary experiments, the one in which interstitial cell stimulation was favored, showed lesser effects, we are inclined to the view that the results are rather dependent upon the gonadotropic factor.

The following theory is offered to explain the facts observed in the foregoing experiments.

In the Marsh-Buffalo strain the breasts of virgins remain undeveloped (scattered ducts and buds) during the early adult life, but the repeated stimulation by the ovarian secretions finally produces a certain degree of acinar development. Up to this point there is no carcinogenesis. Further stimulation, however, carries the anatomical development to a point where the mutation leading to carcinogenesis (a hereditary factor) occurs. This further stimulation occurs because of the failure of the Marsh-Buffalo mouse to produce in later life proper defense mechanism against the repeated stimulation occurring with the recurring ovarian cycles, or because the character of the ovarian secretion changes. If this further stimulation were not due to failure of a defense mechanism or a change in the secretion at middle age then carcinogenesis should occur earlier in the prolan treated mouse which has acinar development 6 months or more before its normal control and still does not develop carcinoma at a rate any faster than its control. Mare's serum hormone and the pituitary hormone stimulation prolong the defense mechanism, possibly by the production of anti-hormones or by maintaining the early ovarian secretion. Massive doses of theelin (sublethal) are required to break down this defense mechanism which functions during early middle life.

SUMMARY

Parenteral administration of prolan, equine gonadotropin, or pituitary gonadotropic preparations produces acinar development of the mammary gland in the young (2 months old) Marsh-Buffalo mouse comparable to that found at the age of one year. Exogenous theelin even in sublethal doses fails to produce this effect.

Sublethal doses of theelin (3.8 mg. per mouse in 5 months) are required to enhance carcinogenesis in the Marsh-Buffalo virgin female mouse. The increase obtained for both the adenocarcinoma of the breast and for lymphosarcoma was only doubtfully significant.

Prolan failed to influence the onset of the adenocarcinoma of the breast or lymphosarcoma in virgin females whether first administered before sexual maturity (720 U per mouse in 12 months), whether first treated at the age of 60 to 90 days (660 U per mouse in 9 months), or whether given in massive doses (2700 U per mouse in 9 months).

Pregnant mare's serum (750 U per mouse in 11 months) and purified pregnant mare's serum hormone (950 U per mouse in 14 months) significantly retarded the onset and decreased the incidence of adenocarcinoma in virgin female mice.

Either subcutaneous or intraperitoneal injections of pituitary gonadotropic hormone preparation over a period of 13 months significantly retarded the onset and decreased the incidence of adenocarcinoma in virgin female mice.

Mice which had received the intermittent doses of prolan, mare's serum preparations and pituitary gonadotropic preparations were able to become pregnant after one year of treatment.

Marsh-Buffalo mice are cancer susceptible and theelin resistant when compared with other cancer susceptible strains of mice.

Acknowledgment is made to Arthur Blanchard for the care of the mouse colony.

We are indebted to Eli Lilly and Company and Parke, Davis & Company for the theelin, and to the Cutter Laboratories for the various mare's serum preparations.

REFERENCES

1. Lacassagne, A.: *Am. J. Cancer* 37: 414. 1939.
2. Bischoff, F., and M. L. Long: *Endocrinology* 23: 327. 1938.
3. Bischoff, F., and M. L. Long: *Am. J. Cancer* 37: 531. 1939.
4. Lewis, A. A., C. W. Turner and E. T. Gomez: *Proc. Soc. Exper. Biol. & Med.* 24: 157. 1939.
5. Suntzeff, V., E. L. Burns, M. Moskop and L. Loeb: *Am. J. Cancer* 27: 229. 1936.
6. Bischoff, F., and L. C. Maxwell: *Am. J. Cancer* 27: 87. 1936.
7. Bischoff, F.: *J. Biol. Chem.* 133: 621. 1940.
8. Bischoff, F.: *Am. J. Physiol.* 121: 765. 1938.
9. Gardner, W. U., A. Kirschbaum and L. C. Strong: *Arch. Path.* 29: 1. 1940.
10. Turner, C. W.: Sex and Internal Secretions. Chapter XI, Second Edition, Allen, Danforth and Doisy. Williams & Wilkins, Baltimore, 1939.
11. Loeb, L., and M. M. Kirtz: *Am. J. Cancer* 36: 56. 1939.
12. Cramer, W., and E. S. Horning: *Lancet* 1: 72. 1938.
13. Jensen, H., and S. Tolksdorf: *J. Biol. Chem.* 133: xiix. 1940.

THE EFFECT OF HYPERPYREXIA UPON SPERMATOZOA COUNTS IN MEN

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IN A SERIES of papers published since 1922, Carl Moore and his coworkers (1, 2, 3, 4) have presented abundant evidence to show that exposure of the testes of the rat, guinea pig, and sheep to temperatures above that of the scrotum results in degeneration of the germinal epithelium and a consequent failure of spermatogenesis. This work was confirmed by Fukui (5) who exposed the scrota of rabbits to midsummer sunlight and found selective destruction of the germinal epithelium. He also applied continuous streams of hot water (44°–49°C.) to the scrota of dogs, cats, guinea pigs, rats and goats and found degenerative changes in the germinal epithelium in every case. Young (4) studied the order of sperm cell degeneration in guinea pig testes after water heated to 46° or 47° C. had been run over the scrotum for 15 or for 30 minutes. He found that degeneration of the germinal epithelium begins immediately after treatment and continues until the end of 12 days. He also studied the effect on reproductive capacity and found that the mating records of the animals showed a marked decline in fertility between the 44th and 77th day after the heat application. Cunningham and Osborne (6) exposed the testes of two rats of known fertility to infra-red rays (48°C.) for 5 minutes and then penned the animals with females. Each was found to be fertile up to about 54 days after which they became sterile for a period of a month. Thus, they confirmed the work of Young. The only negative finding is that of Wilhelm and Schwartz (7) who exposed the testes of 18 guinea pigs to a single short-wave radiation of 20 to 60 minutes duration without finding any gross lesions of the germinal epithelium. They pointed out, however, that physiological changes lessening fertility may have occurred in the absence of demonstrable morphological lesions. The effect of high temperature on spermatogenesis seems general for all mammals but similar experiments on man have not been reported previously. However, in the course of a study on normal human spermatogenesis and the metabolism of human spermatozoa, it was possible to obtain 6 healthy donors who volunteered to undergo a single fever treatment which involved raising of the body temperature from approximately 37°C. to a temperature between 40.5°C. and 41°C. This report will deal with the effect of the elevated body temperature on the total sperm counts at various intervals after the fever treatment.

METHOD

The donors were healthy, young, and unmarried, and their normal total sperm counts, examined at regular intervals, did not vary strikingly from the mean normal counts determined by Hotchkiss (8). Sperm morphology and motility were examined on every specimen, using the dry-smear staining technic of Hotchkiss (8) and the motility method outlined by MacLeod (11). Several specimens were obtained from each donor to establish a normal mean count before the fever treatment. The counts are recorded graphically as the total number of cells in the ejaculate as determined by a hemocytometer count of the number of cells per cc. The fever treatments were given

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in the Physiotherapy Department of New York Hospital under careful clinical supervision.[1] The high temperature within the fever cabinet is induced and maintained through electromagnetic induction from a short-wave apparatus. In addition, the apparatus contains humidifying and air-conditioning systems which maintain a humidity level between 95 and 100% and an adequate circulation of moist air within the cabinet. The cabinet was pre-heated to 43°C. and this temperature was maintained until the patient's oral temperature was approximately 2°C. below the desired fever temperature. In the experiments to be reported, a body temperature of

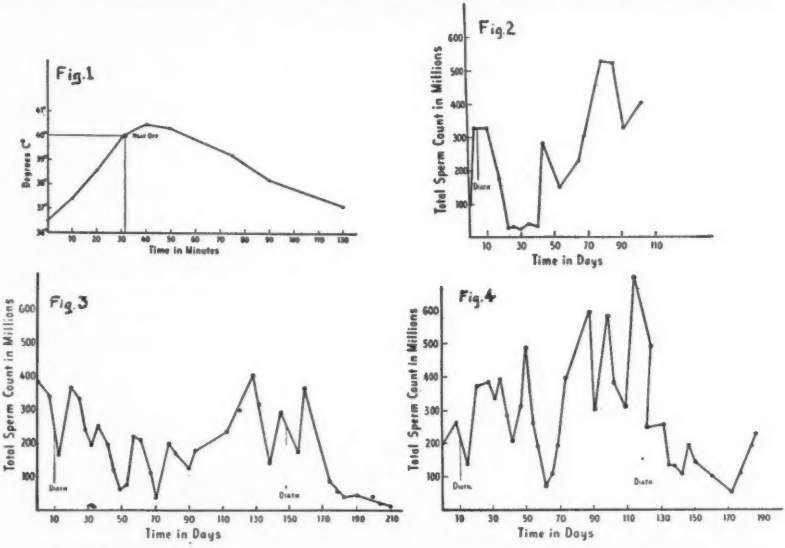

Fig. 1. MEAN BODY TEMPERATURE AFTER EXPOSURE TO HIGH TEMPERATURE. Temperature taken orally until the point at which heat was turned off. Rectal temperature thereafter. Area within rectangle denotes time during which body surface was exposed to environmental temperature of 43°C. Fig. 2 to 7. TOTAL SPERMATOZOA COUNTS OF 3 INDIVIDUALS BEFORE AND AFTER EXPOSURE TO HIGH TEMPERATURE. Individual represented in figure 2 received only one diathermy treatment. DIATH. = time at which treatment was given.

40°C. was considered desirable, though in most cases the maximum body temperature attained was between 40.5°C. and 41°C. Temperatures were taken by mouth until the current was turned off, at which time rectal temperatures were taken until the patient was removed from the cabinet.[2] In these experiments, the nude patient was placed in the pre-heated cabinet with only the head protruding, and his body temperature followed until the desired temperature was reached. The mean time taken to reach this temperature was 45 minutes. Figure 1 shows the mean body temperature of the individuals at intervals after exposure to the high temperatures. It also denotes the mean time at which the current was turned off. The mean chamber temperature from the time the individual was placed inside until the current was shut off was 43°C. (110°F.). This means that for at least 32 minutes the surface of the scrotum was exposed to this high temperature, and that, for a considerably longer period (up to 3 hours), the blood temperature was elevated to points 0.5° to 4°C. above normal.

[1] We are indebted to Dr. Hansen of the Physiotherapy Department for valuable cooperation and to Miss Valentine of that department for indispensable technical assistance.

[2] The rectal temperatures were recorded potentiometrically and could not be taken until the fever apparatus was shut off.

The patient was left in the cabinet until his temperature fell to 39°C. at which time he was taken out and wrapped in blankets until his temperature had returned to normal. As a precaution in post-treatment protection, the patients were hospitalized overnight. Thereafter, at intervals of 3 to 6 days, total spermatozoa counts were taken and the results are recorded below.

RESULTS

Figures 2 to 7 show the total sperm counts of each of 6 individuals before and after a single exposure to high temperatures. It will be seen that the normal counts

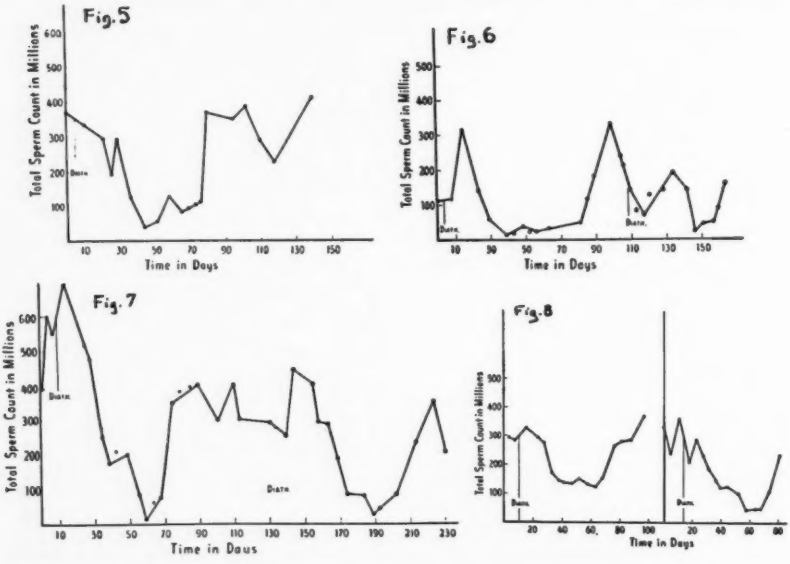

Fig. 5, 6, 7. TOTAL SPERMATOZOA COUNT OF 3 INDIVIDUALS BEFORE AND AFTER EXPOSURE TO HIGH TEMPERATURE.

Fig. 8. COMPOSITE CURVES REPRESENTING THE MEAN TOTAL SPERMATOZOA COUNTS BEFORE AND AFTER EXPOSURE TO HIGH TEMPERATURE. First curve represents mean total counts of 6 individuals. Second curve the mean total counts of 4 individuals.

are subject to considerable variation, and Hotchkiss (8), in a study of normal counts over a period of one year, has shown that such variations are to be expected. However, in every case, it is evident that shortly after the initial fever treatment, the total sperm counts begin to fall, reaching a distinctly low level at intervals ranging from 25 to 55 days after the treatment. Thereafter, this low level is maintained for periods between 15 and 50 days, after which the counts show a relatively rapid rise. In two individuals (fig. 3, 4), the fall in total count after the initial exposure is masked by sporadic rises though the lowest counts recorded are decidedly below the level of the mean counts before the hyperpyrexia.[3]

A second experiment was performed in which 4 of the same individuals of the original group were exposed to a second fever treatment under exactly the same con-

[3] The results shown in figures 3 and 4 are inconclusive though the data are included in the mean values in figure 8. We have no explanation for the sporadic rises and falls in the counts of these individuals after the first exposure to the high temperature, though we believe that the lowest counts recorded are beyond the normal limits of probability. However, the results obtained in these individuals after the second exposure to high temperature are conclusive.

ditions as the first. The data is presented in figures 4 to 7. This experiment confirmed the original findings. It is to be noted that the fall in the counts of the individuals represented in figures 3 and 4 is definite and sustained as compared to the fall after the initial fever treatment.[4] Figure 8 presents a composite curve in which the mean total counts, before and after both fever treatments for all subjects, are given. As would be expected, these curves are considerably smoother and serve to show more clearly and concisely the decided falls and rises in the mean total counts before and after hyperpyrexia. Table 1 summarizes some of the mean time relationships which

TABLE 1

	Mean time for total counts to drop to middle of low level	Mean time for rise to normal level	Period of abnormally low counts
1st Exposure	42 days	25 days	32 days
2nd Exposure	50 days	25 days	37 days

operate. Calculating the time intervals between the hyperpyrexia and the middle o the lowest count level, it will be seen that the mean time for the count to drop to the lowest level after the initial exposure is approximately 42 days. The recovery period is not so easy to calculate but seems to be more rapid (approximately 25 days). After the second treatment, the middle of the lowest count level was reached at about 50 days and the time for recovery is approximately the same as in the first experiment.

DISCUSSION

For obvious reasons, no biopsy material could be obtained in these experiments. Nevertheless, the observations in this report indicate that the effect of high temperatures on spermatogenesis in man follows quite closely the results of similar experiments on animals, and indeed, that the time relationships between the application of the heat and the resulting effect on spermatogenesis are comparable in both cases. For approximately 18 days after the fever, the human total sperm counts remain at a relatively normal level which indicates that the store of mature spermatozoa in the epididymis and ductus deferens is of such magnitude that any injury to the germinal epithelium is not manifested immediately. In the guinea pig, Young (4) has shown that the degeneration of the germinal epithelium begins immediately after application of the heat and continues for about 12 days at which time there is almost complete evacuation of the germinal epithelium. He also showed that the mature sperm in the epididymis were very resistant to heat injury and this observation is confirmed in man, since we have not observed any marked changes in the motility or morphology of the spermatozoa after hyperpyrexia. Also, Young (4) for the guinea pig, and Cunningham (6) for the rat, have shown that the reproductive capacity of those animals is markedly affected (or absent) between 44 and approximately 80 days after heat injury of the testes. In like manner, the lowest level in the human sperm counts is reached between 44 and 50 days after hyperpyrexia and is maintained for periods ranging from 14 to 30 days. Although we cannot conclude that, in these experiments, the lowest count level represents a period of sterility, it should be pointed out that Macomber and Saunders (10), in a statistical study on the relation between the human spermatozoa count and fertility, come to the conclusion "that although pregnancy is not impossible with a count below 60 million spermatozoa per cc., the chances are

[4] This individual left for the summer while his spermatozoa count was still exceedingly low. A count was made 3 months later at which time it was evident that it had returned to normal. The total count was 475 million.

slight without an increase in numbers." In 53 cases studied, only 5 with counts below 60 million per cc. were fertile. Furthermore, they concluded that the count per cc. is the important factor and not the total number of cells present in the specimen. On the basis of such evidence, most of the individuals in our experiments had impaired fertility between approximately the 40th and 70th days after the hyperpyrexia.

A number of important clinical inferences are suggested by this study. Febrile states are capable of materially impairing normal spermatogenesis (11). The effect upon the testes may be entirely independent of the bacteria or their by-products and, rather, may be directly related to the duration of the elevation of body temperature. Mills (12) describes pathological changes in the testes in epidemic pneumonia, which were independent of the infecting organism and varied in severity with the total length of the illness. Typical of the pathological changes were cessation of spermatogenesis, and degeneration of preformed spermatocytes, spermatids, and spermatozoa. He concluded that, in the absence of definite evidence to the contrary, the source of the testicular damage (which seemed to be confined to the spermatogenetic tissue) lay in the circulating toxins. However, at the time of this study, nothing was known of the effects of hyperpyrexia upon testicular function, and it is possible, indeed, that many of the effects noted by Mills were due, not to circulating toxins but to prolonged elevation of body temperature.

The immediate remote effects of prolonged elevations in body temperature or of repeated febrile shocks can only be surmised. It is evident, however, that single specimens of semen should not be accepted as incontrovertible evidence of impaired fertility. A second or third examination should be made 25 or more days later. It is to be noted that approximately 67 days elapsed between the time of injury and the return of the total spermatozoa count to a normal level. Conversely, it may be reasoned that a similar interval of time, at least, is to be expected between stimulation of the germinal epithelium (by hormonal or other methods) and a consequent elevation of the mature spermatozoa count.

SUMMARY

Elevation of body temperature in man in conjunction with a simultaneous elevation of the environmental temperature results in a marked drop in spermatozoa production. The effect of hyperpyrexia upon spermatogenesis does not become apparent for nearly 3 weeks and may last for a period of 50 days thereafter. The clinical inferences of this work are discussed.

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REFERENCES

1. Moore, C. R., and R. Oslund: *Am. J. Physiol.* 67: 595. 1924.
2. Moore, C. R., and W. J. Quick: *Am. J. Physiol.* 68: 7. 1924.
3. Moore, C. R.: *Am. J. Anat.* 34: 337. 1924.
4. Young, W. C.: *J. Exper. Zool.* 49: 459. 1927.
5. Fukui, N.: *Japan Medical World* 3: 27. 1923.
6. Cunningham, B., and J. Osborn: *Endocrinology* 13: 93. 1929.
7. Wilhelm, S. F., and A. M. Schwartz: *J. Urol.* 40: 335. 1938.
8. Hotchkiss, R.: *N. Y. State J. of Med.* In Press.
9. MacLeod, J.: In manuscript.
10. Macomber, D., and M. B. Saunders: *New Eng. J. Med.* 200: 981. 1929.
11. Meaker, S. R.: Human Sterility. The Williams & Wilkins Co., Baltimore, 1934.
12. Mills, R. G.: *J. Exper. Med.* 30: 505, 1919.

THE EFFECT OF THYROXIN ON GROWTH, OXYGEN CONSUMPTION AND BODY COMPOSITION OF HEREDITARY DWARF MICE[1]

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THE MUTATION causing dwarfism in mice was found by Smith and MacDowell (1, 2) to act primarily on the pituitary and indirectly on the endocrine glands which it controls. Pituitaries from dwarf mice implanted into other dwarfs stimulated sexual development but not growth. Normal pituitaries and crude pituitary extracts, however, produced both effects. The dwarf mouse has a low metabolic rate (3, 4, 5) and an atrophic thyroid (1, 6). Snell (6) was the first to observe growth stimulation in dwarf mice treated with thyroid. The growth resulting is reported (7) to be abnormal as the ears and hair are overemphasized and the apparent growth exaggerated by large masses of fat. Following 140 days of treatment all of 30 dwarf mice had body weights between 20 and 30 gm. (7). Treatment for shorter periods results in only moderate growth with no obvious excess fat (8, 9). The normal occurrence of fat dwarf mice with body weights up to 34 gm. was reported by Boettiger and Osborn (10). They found that considerable growth occurs in untreated animals following a critical period between the 17th and the 35th day of age during which 16% of the body weight is lost and then regained. Average body weights of 12 to 14 gm. were finally reached. Certain of these untreated dwarfs grew as well as thyroid treated dwarf mice (8, 9).

A further study made in an effort to reduce the great variability in growth found to result from differences in the lengths of the critical periods (10) led to the conclusion that in the dwarf mouse the temperature controlling mechanism develops slowly and body temperature is not maintained for some time after weaning has begun (5). Dwarfs placed at 33°C. before the critical period lost no weight and grew more rapidly than the average dwarf mouse although the maximum weight attained was not greater. These observations on the effect of environmental temperature suggested a re-examination of the relation of the thyroid gland to growth in dwarf mice. In this work the oxygen consumption of the dwarf was first measured, the amount of thyroxin necessary to raise the oxygen consumption to a normal level determined, and finally the effect of this dosage on growth and body composition was observed.

MATERIALS AND METHODS

The origin of our dwarf strain and the treatment of animals in our colony have been described (10). Oxygen consumption was measured at 33°C. by a method devised for these studies using the principle of the constant pressure manometer (11).

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[1] A portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Harvard University.

The mouse was placed in a constant temperature room at 33°C., without food, 12 to 18 hours before measurements were made. Oxygen consumption was determined for 2-minute periods during which the animal was perfectly quiet. Surface area was calculated by the formula of Benedict and Lee (3).

In all experiments recorded here thyroxyl tablets[2] containing a partially purified sodium salt of thyroxin were used. The tablets were carefully ground in water so that each tenth of a cubic centimeter contained the amount to be given. This was placed directly in the animal's stomach with a hypodermic syringe and needle the tip of which was rounded off with a rim of solder.

To determine body composition the total carcass weight of the animal was found immediately after removal of the intestinal contents. By noting the change in weight

TABLE 1. THE OXYGEN CONSUMPTION OF DWARF AND NORMAL MICE

No. of animals	Average age	Average weight	O_2/gm./24 hr.		O_2/m.²/24 hr.	
	days	*gm.*	*cc.*	*St. Dev.* ±	*l.*	*St. Dev.* ±
			Dwarf Mice			
15	15	5.77	35.7	3.6	70.8	5.6
9	19	5.76	29.4	2.8	58.3	5.3
12	22	5.42	31.3	3.8	61.0	3.8
10	25	5.96	30.0	3.6	60.2	6.8
9	33	6.13	34.0	4.5	68.9	8.1
11	40	6.30	36.4	8.3	74.3	18.0
9	50	7.00	32.3	4.5	68.5	8.9
13	100	10.80	34.0	3.2	83.6	7.2
			Normal Mice			
3	3	2.37	48.6		68.9	
1	5	3.59	37.4		64.9	
1	14	7.09	54.1		115.7	
3	19	8.32	56.2		127.9	

of the carcass after drying to constant weight the amount of water was calculated. The fat was measured after Soxhlet extraction with ether and the total nitrogen by Kjeldahl determination on a dry defatted sample. Percentage composition was calculated on the basis of the fat-free weight obtained by subtracting the fat content from the wet carcass weight.

The Oxygen Consumption of Dwarf and Normal Mice

Benedict and Lee (3) studied the basic metabolism of various breeds of mice including a few dwarf mice of the black silver strain. The metabolic rate of the 6 to 8 gm. dwarf was 40% per unit surface area below that of normal adult albino mice. In the present study dwarf mice of various ages were used. The animals were divided into 8 age groups and an average age assigned to each group. The results of 87 determinations on dwarf mice and of 8 on normals is summarized in table 1. The standard deviations are rather high in the case of the dwarf mice. As the range of body weights in all groups was extreme this variation is not surprising and reflects the fact that dwarf mice of the same age may not be in the same physiological state. If an R.Q. of 0.72 is assumed the 19-day-old normal mouse has a basal metabolism of 601 cal./m²/24 hours, a value comparing well with most of the published work on adult normal mice (12) with the notable exception of the very low values found by Benedict and Lee (3).

[2] Obtained from Squibb & Sons.

Until the 7th day of life there is little difference between dwarf and normal mice in body weight or metabolic rate. The O_2 consumption of these young dwarf and normal mice is about 60 l./m²/24 hours measured in a modified Warburg at 31°C. (13). From the 15th to the 19th day the critical period weight loss of the dwarf is paralleled by a 17% drop in O_2 consumption (table 1). As averages may be misleading when dealing with this type of data repeated determinations on the same dwarf were made in 4 cases during early life. In every case there was a fall in basal O_2 consumption which averaged 20%, although in no case was it certain that a reading had been made at the lowest value attained. The slow increase in O_2 consumption per unit surface area following the critical period decline is accompanied by a weight increase.

The normal mouse grows markedly in contrast to the behavior of the dwarf, and

TABLE 2. THE EFFECT OF THYROXIN ON THE OXYGEN CONSUMPTION OF DWARF MICE

Animal	Average weight	Daily dosage	O_2/gm./24 hr. Initial	Final
	gm.	γ	cc.	cc.
*	10.8	0	34.0	34.0
1	13.7	2.5	32.8	41.2
				39.6
				39.3
2	10.2	2.5	38.1	48.1
			39.3	52.6
				46.5
Av.	12.0	2.5	36.7	44.9
3	13.9	8.0	35.5	50.8
				59.7
4	11.0	8.0	39.5	55.6
			37.7	60.5
Av.	12.5	8.0	37.6	56.7
5	9.6	12.5		67.2
	8.7	12.5		66.3
	14.8	12.5		57.1
Av.	11.0	12.5		63.5

* See Table 1.

has a rapidly increasing basal O_2 consumption during the second week of life (table 1). This fact makes the difference in O_2 consumption between dwarf and normal mice very evident in later life. Twelve dwarf mice with an average body weight of 10.8 gm. had an O_2 consumption per unit surface area 35% below that of 3 normals whose average weight was 8.3 gm. It is interesting to note that the result of this comparison between animals of about the same weight closely corresponds to the difference found by Benedict and Lee (3) between dwarfs and adult normals. As our dwarfs are the result of outcrosses from the original black silver strain it is important to find that their metabolism is reduced to the same extent as that of the pure strain dwarf.

The Relation Between Oxygen Consumption and the Dose of Thyroxin

The amounts of thyroid powder and of thyroxin that have been used on dwarf mice by various workers range up to 20γ of thyroxin per day (9). Although little data has been published the growth resulting in all cases was similar for short periods of treatment. Preliminary experiments showed the optimum dosage to be about 10γ per day when given in the manner described. An experiment was then set up to test the effect on O_2 consumption of 3 different doses near this optimum (table 2). Adult

dwarf mice were used and in 4 out of 7 animals O_2 consumption was determined both before and after treatment. One to three measurements, made at different times, established each value. As there is a considerable latent period before the full response to thyroxin is obtained, final determinations were made after about 14 days of treatment.

When plotted these values give a logarithmic curve relating dose to rate of O_2 consumption. The basal O_2 consumption of normal mice weighing 8.3 gm. was found to be 56.2 cc./gm./24 hours. This rate corresponds to that of dwarf mice receiving 8γ of thyroxin in tablet form per day.

In the following experiments on growth a somewhat higher dose than that necessary to bring the basal O_2 consumption to normal was used. To animals above 5 gm. body weight, 25γ were given on alternate days while animals below 5 gm. received one-half this amount. Table 3 gives the O_2 consumption of dwarf mice of various weights under treatment with this standard dose of thyroxin. These values average slightly higher (about 10%) than those found in normal mice of 8.3 gm. body weight. Much higher doses, however, may be tolerated. One dwarf of 12.5 gm. received 50γ per day for 10 days and 100γ per day for 6 subsequent days. At the end of this period the animal's weight was 12.1 gm. and its O_2 consumption 30% above that of normal mice. This tolerance to thyroxin is in striking contrast to the sensitivity of normal mice to thyroxin after the removal of the thyroid.

TABLE 3. THE OXYGEN CONSUMPTION OF DWARF MICE RECEIVING THE STANDARD DOSE OF THYROXIN, 25γ ON ALTERNATE DAYS

Body weight	O_2/gm./24 hr.	O_2/m.²/24 hr.	*Cal./m.²/24 hr.
gm.	cc.	l.	*calories*
5.10	72.3	139.8	657
5.70	74.0	146.2	687
5.79	46.4	93.0	437
7.46	63.2	136.0	639
7.61	69.6	152.8	718
7.75	67.4	148.0	696
8.32	62.3	141.2	664
9.74	55.1	130.8	615

* R.Q. of 0.72 is assumed.

The Growth and Body Composition of Thyroxin-Treated Dwarf Mice

The average growth curves of thyroxin treated and untreated dwarf mice are shown in figure 1. Treatment was begun on 11 animals when less than 15 days of age. Three of these were given continuous treatment for 155 days, at the end of which period their average weight was 13.8 gm. The others were treated for about 17 days, a sufficient length of time to cover the critical period. In contrast to the control group the treated dwarf shows little or no decrease in weight at weaning, though a definite slowing of growth does occur. The difference in weight between treated and control of the same age is due mainly to the effect of thyroxin in shortening the critical period. Ultimately the same maximum weight is reached.

When the body temperature of young dwarf mice is maintained by a high environmental temperature, no weight loss occurs at the time of the critical period and growth is continued to a 12 to 14 gm. maximum at a rate equivalent to that of thyroxin treated dwarf mice. Figure 2 shows the growth of our fastest growing dwarf and of the average dwarf compared with dwarf mice raised at a high environmental temperature

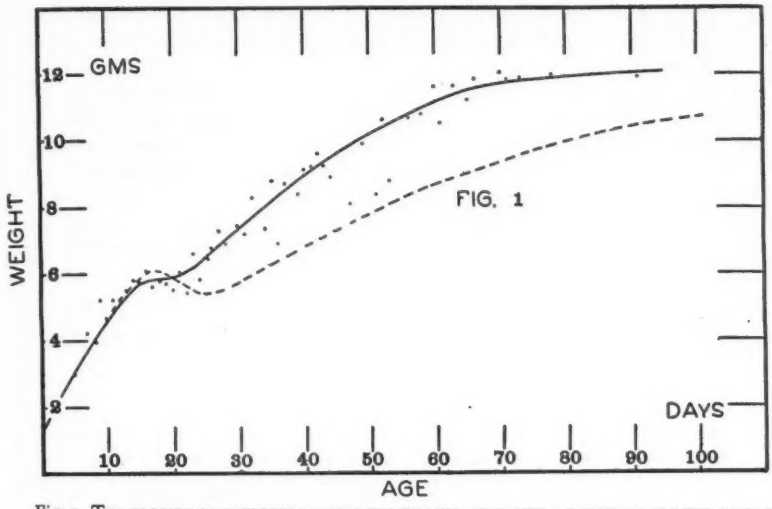

Fig. 1. THE GROWTH OF THYROXIN TREATED DWARF MICE, SOLID LINE, COMPARED WITH THE AVERAGE DWARF GROWTH CURVE, DOTTED LINE.

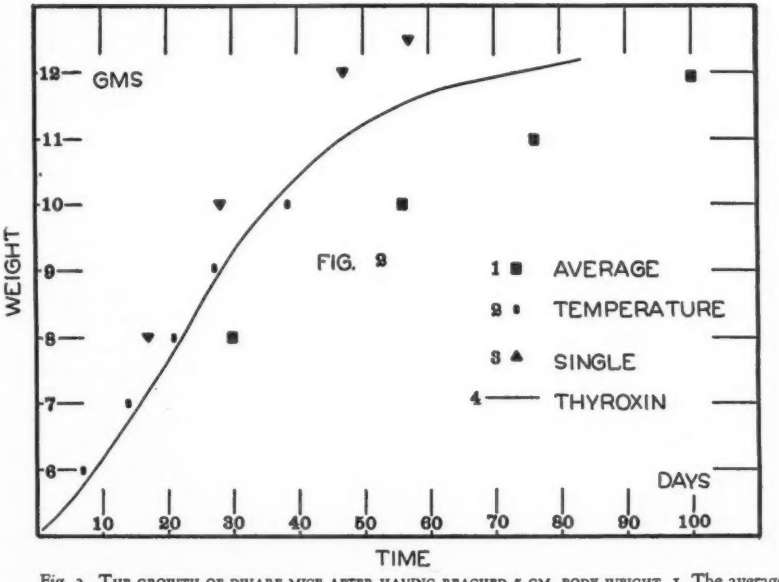

Fig. 2. THE GROWTH OF DWARF MICE AFTER HAVING REACHED 5 GM. BODY WEIGHT. 1. The average dwarf growth following the weight loss. 2. The growth of 6 dwarf mice at 33°C. 3. A single dwarf growth curve, the most rapidly growing animal studied. 4. The growth of dwarf mice treated with thyroxin beginning from the 13th to the 15th day of age.

and dwarfs treated with thyroxin. Zero time was taken in every case when a weight of 5 gm. had been reached, and for the average dwarf the growth shown is that occurring after the weight loss of the critical period. The rapid growth of the single

dwarf is due in part to the accumulation of fat, while the slow growth of the average animal results from the inclusion of many small dwarfs unable to maintain their body temperature.

Dwarf mice all through life have a higher percentage of fat than normals (table 4). Thyroxin reduces this fat content to the level found in normal mice. Older dwarfs, even when their metabolic rate is raised to normal by thyroxin, may still accumulate fat up to 40% of the fat-free weight. The percentage of total nitrogen increases between the 10th and 20th day in both dwarf and normal in spite of the great difference in growth rate during this period. This indicates that the age at which chemical maturity is reached is the same in both, although it is possible that this parallelism merely

TABLE 4. BODY COMPOSITION

No. of animals	Age	Weight		Per cent of fat-free weight		
		Whole[1]	Fat-free	T.N.	Fat	H_2O
	days	*gm.*	*gm.*	%	%	%
			Dwarf Mice			
5	10	5.2	4.103	2.5	19.1	79.7
7	20	6.2	4.763	3.0	20.6	75.5
5	29	5.6	4.448	3.1	20.1	74.3
4	58	8.1	6.689	3.2	13.8	73.7
5	120	11.8	8.807	3.2	22.7	73.1
3	200	15.4	10.400		48.0	
			Normal Mice			
5	10	6.1	5.054	2.6	14.2	79.6
2	19	10.5	8.946	3.0	12.0	75.2
3	29	13.4	11.322	3.1	6.9	74.4
1	35	15.0	13.758	3.2	6.2	75.4
			Thyroxin Treated Dwarf Mice[2]			
3	35	8.1	6.971	3.1	8.8	74.9
1	175	10.0	7.500		7.2	71.1
1	175	15.5	9.520		40.3	72.9

[1] Weight before animal was killed and intestinal contents removed.
[2] Treatment begun on the 13th day of age.

reflects the change in diet at weaning. The decrease in percentage of body water also follows age more closely than weight and is another sign of maturity in body composition. The maximum fat-free weight found in untreated dwarf mice was 10.4 gm. at 200 days and for thyroxin treated, 9.5 gm. at 175 days. This demonstrates that administered thyroxin does not increase the maximum fat-free weight that may be attained by dwarf mice. The adult dwarf corresponds in fat-free weight, as in ossification (10), to a 4 to 5 weeks old normal animal.

DISCUSSION

The effect of thyroid on the growth of dwarf mice presents a peculiar problem because of the reported excessive fatty deposits occurring after prolonged treatment (7). Throughout life untreated dwarf mice have a higher percentage of body fat than normal mice. Certain older dwarfs accumulate very large fat reserves. During the active growth period dwarfs fed thyroxin in amounts sufficient to bring their basal oxygen consumption to normal have a low fat content similar to that of normal mice. The large increases in fat following prolonged thyroid administration are therefore not specifically due to a thyroid growth effect but possibly to some secondary result such as improved intestinal absorption.

The critical period in the dwarf growth curve is practically abolished when thyroxin administration is begun before the 15th day. The difference in weight between thyroxin treated and the control animals of the same age is mainly the result of this shortening of the critical period. Dwarf mice maintained at a high environmental temperature grow at the same rate as thyroxin treated dwarfs and they also do not have appreciable critical periods. The ability to control body temperature develops slowly in the dwarf (5). As all animals were placed at the same environmental temperature 12 to 18 hours previous to measurement of their oxygen consumption, all were subject to the same heat loss. The fact that under these identical conditions animals during the critical period were unable to raise their metabolism and presumably their body temperature to as high a level as at other times indicates that between the 15th and the 19th day some fundamental shift fails to occur. The failure of the metabolic rate to increase in the dwarf as it does in the normal mouse at this time supports this idea. At the 15th day there is commonly observed a break in the normal mouse growth curve (14) associated with the change to a solid diet and the inability of milk to furnish the tremendous energy requirements of this period of rapid growth. The dwarf is now unable to furnish even the minimum demands of its metabolism through the absence of some pituitary factors. Only a high external temperature will prevent a lowering body temperature with resulting weight loss.

The effect of thyroxin on the critical period and the growth rate of the dwarf mice is apparently due mainly to the elevation of the body temperature by an increased metabolic rate rather than to any growth promoting property. In support of this view it is found that 2,4-dinitrophenol raises the body temperature of critical period dwarf mice and lessens the weight loss, and that growth at a high environmental temperature is as great as that following thyroxin treatment.

Although the present study indicates that administered thyroxin while raising the metabolic rate to normal has no specific growth effect on our dwarf mice we must still consider the possibility of secretion from the animal's own thyroid gland. Salmon (15) has shown in the rat that the absence of the thyroid from birth prevents any growth response from pituitary implants. From her results one may conclude tentatively that rats may grow to about 30 gm. body weight in the absence of the pituitary growth principle and of appreciable thyroxin. This growh is almost normal during the first 7 days and reaches a plateau at the time of weaning which, however, is not associated with the withdrawal of milk. Boettiger and Osborn (10) found that dwarf mice were recognizable by the seventh day and that the critical period began at weaning. This striking parallelism between dwarf mice and rats thyroidectomized at birth is further emphasized by a close similarity in appearance. When rats are thyroidectomized on the 7th day typical cretin features did not appear (16). The dwarf then probably suffers from an atrophic thyroid some time before this deficiency can be recognized either by physical appearance or basal metabolic rate.

Although no conclusive answer can be given at this time the data available indicate the dwarf thyroid to be inactive throughout life. A study of the dwarf mouse substantiates further the mass of experimental evidence showing the effect of the thyroid on growth to be secondary to the growth promoting property of the pituitary.

SUMMARY

The oxygen consumption of dwarf mice is 30% to 40% below that of normal mice of approximately the same weight. This is the result of the failure of the metabolism of the dwarf to increase during the second week when that of the normal nearly doubles.

The critical period weight decrease is paralleled by a 17% drop in oxygen consumption and an inability to regulate body temperature.

Older dwarf mice given 8γ of thyroxin per day by mouth have an oxygen consumption similar to that of normal mice. The equivalent of 12.5γ per day given to dwarf mice before the critical period prevents the weight loss characteristic of this time and growth continues at a rate found in postcritical untreated dwarf mice.

A high environmental temperature and thyroxin both produce the same rate of growth in dwarf mice and neither results in greater final weights than are normally found in untreated animals.

Dwarf mice have a high fat content throughout life but calculated on the basis of fat-free weight the concentration of total nitrogen and water are normal for the age of the animal.

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REFERENCES

1. Smith, P. E., and E. C. MacDowell: *Anat. Rec.* 46: 249. 1930.
2. Smith, P. E., and E. C. MacDowell: *Anat. Rec.* 50: 85. 1931.
3. Benedict, F. G., and R. C. Lee: *Ann. de Physiologie* 12: 983. 1936.
4. Kemp, T.: *Acta Pathol. et Microbiol. Scand. suppl.* 37: 290. 1938.
5. Boettiger, E. G.; *Proc. Am. Physiol. Soc.* 129: 16. 1940.
6. Snell, G. D.; *Anat. Rec.* 47: 316, 1930.
7. MacDowell, E. C., and T. Laanes: Carnegie Inst. Wash. Yearbook. 31: 47. 1932.
8. Bates, R. W., T. Laanes and O. Riddle: *Proc. Soc. Exper. Biol. & Med.* 33: 446. 1932.
9. Kemp, T., and L. Marx: *Acta. Pathol. et. Microbiol. Scand.* 13: 512. 1936.
10. Boettiger, E. and C. M. Osborn: *Endocrinology* 22: 447. 1938.
11. Boettiger, E. G.: *Nature* 142: 151. 1938.
12. Benedict, F. G., and E. L. Fox: *Arch. f. d. ges. Physiol.* 231: 455. 1933.
13. Boettiger, E. G., and C. M. Pomerat: Unpublished data.
14. MacDowell, E. C., W. H. Gates and C. G. MacDowell: *J. Gen. Physiol.* 13: 529. 1930.
15. Salmon, T. N.: *Endocrinology* 23: 446. 1938.
16. Salmon, T. N.: *Proc. Soc. Exper. Biol. & Med.* 35: 489. 1936.

BROMINE AND THE THYROID[1]

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BROMINE has often been thought to play an important part in the physiology of the thyroid, as well as in that of other endocrine organs. The interesting observation that thyroid contains more Br than any other tissue has been made by Bernhardt and Ucko (1), Neufeld (2) and others. Moreover, Tanino (3) claims that human thyroids with a small amount of colloid have less Br than the normal gland. Toxopeus (4) and Kuranami (5) state that blood Br rises following the feeding of desiccated thyroid. However, many of these and similar findings were made by the use of the now discredited Br method of Pincussen and Roman. The present work is an attempt to obtain more specific evidence of the relation of Br to the thyroid.

METHOD

Bromine was estimated by the method of Leipert and Watzlawek (6). Two cc. samples of blood were used for most analyses. A second analysis can be made with the same silver chromate sulfuric acid mixture by simply changing the receiver. Since the special apparatus is adapted for the use of solutions only, tissues were digested to a thickish fluid with strong caustic, before introduction into the reaction vessel. The method was checked on pure Br solutions and also by recovery experiments; we find it meets fully the claims of its authors for accuracy.

Influence of Diet on Blood Br

The experiments were conducted mostly on rabbits, whose daily ration consisted of 35 gm. of alfalfa and 35 gm. of oats, supplemented by greens twice weekly. On this diet the blood Br usually varied between 2 and 3 mg. %, a figure considerably higher than those reported for man, dog and other animals. The cause of our high values was found in the low Cl content of the diet. Since the kidneys excrete Cl preferentially over Br (7), blood Br would tend to rise on this low Cl diet. In support of this hypothesis, the following observations are submitted.

(*a*) Bromine and Cl analyses of the foods are given showing their low Cl content.

	Cl	Br
	mg.per 100 gm.	mg.per 100 gm.
Alfalfa hay	81.4	1.28
Rolled oats	54.1	0.85
Whole oats		1.72

(*b*) Blood Br was readily lowered by adding NaCl or KCl to the diet but not by sodium citrate. A few examples are shown in table 1.

(*c*) On a daily diet of 125 gm. of carrots and 35 gm. of oats, which contains 50% more Cl than does the stock diet, blood Br was 1.2–1.5 mg.%.

The variations of blood Br on a constant diet are not more than 0.3 mg.%.

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Effect of Thyroid Administration on Blood Br

Rabbits whose blood Br was constant for a week or more were fed a total of 0.6 to 1.2 gm. of desiccated thyroid over a period of a week. Instead of the increase reported by Toxopeus and by Kuranami, we find a decrease with each of the diets used and irrespective of the initial blood Br level. However, the fall did not occur until 2 to 3 weeks after medication, following which the blood Br slowly returned to its previous level. Examples of the 8 animals in which this experiment was made are given in table

TABLE 1. INFLUENCE OF FEEDING VARIOUS SALTS ON BLOOD BROMINE

No. and sex	82 M	83 F	75 M	85 F	75M	78F	80 M
Daily medication	100 mg. NaCl	100 mg. NaCl	250 mg. NaCl	500 mg. NaCl	170 mg. Na citrate	500 mg. KCl	500 mg. KCl
	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br
Control	2.34	2.56	3.43	3.88	2.80	2.66	2.96
2 weeks after	1.37	1.86	2.37	2.07	2.88	1.27	1.30
3 weeks after			1.31	1.48			
4 weeks after	1.34	1.77			3.06		

2 along with 2 controls. The dose of thyroid used caused a weight loss of 50 to 200 gm. In no case was there a rise in blood Br.

Effect of KI *on Blood* Br *of Goitrous Rabbits*

When rabbits with hypertrophied thyroids are given a little iodine, the glands will involute; an excessive thyroid secretion will take place resulting in a considerable loss of body weight. If the gland is not too large, the rabbit will regain control and its metabolism will return to normal. But when the thyroid weighs several grams (normal weight 0.15–0.25 gm.) and iodine is given, the animal may die of intense hyperthyroidism (8). Under these circumstances it would excrete far more thyroid hormone than that given to the rabbits shown in table 2.

Table 3 gives the data of an experiment in which 3 rabbits with large goiters were injected subcutaneously with 2.5 mg. of KI every third day for 2 weeks after the right thyroid lobes had been removed for Br analysis and histological examination. *Rabbit* 56 had an enlarged thyroid that contained a small amount of colloid from a former medi-

TABLE 2. EFFECT OF THYROID FEEDING ON BLOOD BROMINE

No. and Sex	68 F	79 M	69 F	70 M	23 M	54 M	35 M
Diet[1]	A & O	A & O	A & O	A & O	A & O and 0.125 gm. NaCl	A & O and 0.125 gm. NaCl	Carrots, oats and 0.125 gm. NaCl
Thyroid given	None	None	0.6 g.	0.6 g.	0.9 g.	0.9 g.	0.9 g.
	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br	mg. % Br
Av. control level[2]	3.20	3.04	3.15	3.03	1.88	1.91	1.37
1 wk. after thyroid	3.02	2.90	2.76	2.67	1.57	1.67	1.01
2 wks. after thyroid	2.96	2.88	2.72	2.69			
3 wks. after thyroid	2.78	2.85	2.65	2.69	1.26	1.32	0.65
4 wks. after thyroid	2.84	2.92	2.77	2.72	1.32	1.54	0.74
6 wks. after thyroid	2.86	3.05	2.79	2.75			
7 wks. after thyroid	2.78	2.98	2.99	2.78	1.36	1.62	1.81

[1] A & O = alfalfa and oats.

[2] The blood bromine had been stabilized for 2 to 4 weeks previous to feeding thyroid and the figures given as control levels are averages of 2 to 4 observations on different days.

cation with iodine and desiccated thyroid; it was again undergoing hyperplasia. As a consequence of the administration of KI the rabbit lost 120 gm. in weight in a week. The thyroids of the other 2 rabbits were also greatly enlarged but markedly hyperplastic, and the animals showed far more intense reactions of severe hyperthyroidism. *Rabbit* 50 lost 300 gm. and animal 66, 530 gm. in weight in 10 days. They would probably have died had the left lobes not been removed, since other goitrous rabbits similarly treated with I have succumbed. Two weeks later the left lobes were removed for Br analysis and histological examination. At this time the left lobes of all 3 rabbits proved to be firm, translucent and completely involuted. The blood Br was determined at short intervals during the entire experiment.

Table 3 shows a gradual fall to a minimum of 35 to 50% of the starting Br level and as in the experiments where thyroid was fed, the decrease did not show up until nearly 2 weeks after iodine administration. The lowering of blood Br either by feeding

TABLE 3. EFFECT OF IODIZING GOITROUS RABBITS ON BLOOD BROMINE

Date	50 mg.%	56 mg.%	66 mg.%	Treatment
5-14	3.29	3.39	3.33	Removed R. thyroid lobes
5-16	3.07	3.05	3.37	
5-20	2.99	3.14	3.21	
5-23	2.91	3.21	3.13	5-20, 5-22, 5-25, 5-28, 5-31, 2.5 mg. KI subcut.
5-27	3.05	3.10	3.26	
5-31	2.78	2.94	2.83	
6-3	2.48	2.43	2.36	
6-10	1.83	2.02	1.97	6-4 Removed L. thyroid lobes
6-17	1.90	2.10	1.78	
6-24	1.87	2.24	1.68	
7-8	1.98	2.67	1.83	
8-5	2.15	3.06	2.33	

thyroid or by the excessive thyroid secretion of iodized goitrous rabbits can be ascribed to the well known diuretic and stimulating action of thyroid on the kidney. Under the influence of thyroid greater amounts of bromides as well as of other salts would be excreted and so lower the concentration of Br in the tissues. To verify this explanation, a metabolism experiment was made on 4 rabbits, 2 of which served as controls, to determine the Br balance on administration of thyroid. There was a retention in the control animals and in the fore period of the experimental animals of about 0.15 mg. per day, while in the case of the 2 rabbits receiving 0.6 gm. of desiccated thyroid, the positive balance of 0.15 mg. per day became a negative balance of 0.15 and 0.25 mg. respectively for a period of a week after thyroid was fed. (The feces were not analyzed—most of the Br is found in the urine.) This loss is sufficient to account for the lowering of blood Br after thyroid medication.

Significance of High Thyroid Br *Concentration*

Bromine analyses of the thyroids and blood are given in table 4. The blood was taken shortly before the rabbits were anesthetized with ether for thyroid removal. The control (right) thyroid lobes of 50 and 66 that were markedly hyperplastic contained about 40% more Br than the blood taken at the same time. This agrees with the many reports, some of which have been cited, that show more Br in thyroid than in any other tissue. Similar relations between thyroid and blood Br were found in 2 additional rabbits. A moderately active calf thyroid had 2.7 mg. Br per 100 gm., while the thymus of the same animal contained only 0.7 mg.% of Br. In *Rabbit* 56 whose thyroid showed early hyperplasia, having a moderate amount of colloid, the blood and thyroid Br were about equal.

The left lobes, removed 2 weeks after iodine administration was begun, had returned to the resting colloid stage, and had a Br content only 41 to 68% of that of the right lobes. Blood Br had decreased to about 3/4 of the control value so that the thyroid Br was now somewhat lower than the blood Br in all cases. In other words hyperplastic thyroid tissue of low iodine and colloid content has more Br than the circulating blood, while in fully iodized thyroids, rich in colloid, no more Br is found than is present in the blood. Tanino's findings are just the reverse of ours, namely thyroid glands (of man) with little colloid have little Br, while those having much colloid contain more Br. He gives the iodine content of these glands as well, but the I values do not correspond to the colloid content. So while the thyroid, under certain conditions contains more Br than blood, our experience leads us to conclude that in general thyroid Br bears a direct relation to tissue Br.

TABLE 4. EFFECT OF IODIZING GOITROUS RABBITS ON THYROID BROMINE

56			50			66			
R. thyroid lobe wt.	R. thyroid lobe Br.	Blood Br	R. thyroid lobe wt.	R. thyroid lobe Br	Blood Br	R. thyroid lobe wt.	R. thyroid lobe Br	Blood Br	
gm.	mg. %	mg. %	gm.	mg. %	mg. %	gm.	mg. %	mg. %	
1.27	3.08	3.39	0.58	4.63	3.29	0.93	4.74	3.43	Control (Before iodine)
L. thyroid lobe			L. thyroid lobe			L. thyroid lobe			
1.10	2.14	2.61	0.34	1.87	2.50	0.49	2.60	2.34	After iodine

Rabbit 56, thyroid early hyperplasia.
Rabbits 50 and 66, thyroid marked hyperplasia.

The high Br content of thyroid may be explained on the assumption that thyroid tissue can distinguish only imperfectly between iodine and bromine. Glands which are hyperplastic due to a relative or absolute deficiency of iodine, will in the absence of I seize Br instead. (There is 10 to 100 times as much Br as I in foods and water.) When iodine is supplied to animals with hyperplastic thyroids, they quickly lose the Br which has been accumulated in a futile effort to make good the lack of iodine.

SUMMARY

Thyroid administration or excessive thyroid secretion brings about a lowering of blood Br probably due to the diuretic and stimulating action of thyroid on the kidney. Hyperplastic thyroid contains more Br than does blood of the same animal while the resting colloid containing gland has no more Br than the blood.

REFERENCES

1. BERNHARDT, H., AND H. UCKO: *Biochem. Ztschr.* 170: 459. 1926.
2. NEUFELD, A. H.: *Canadian J. Res.* 14B: 160. 1936.
3. TANINO, F.: *Biochem. Ztschr.* 241: 392. 1931.
4. TOXOPEUS, M. A. B.: *Arch. exper. Path. u. Pharmacol.* 154: 247. 1930.
5. KURANAMI, T.: *J. Biochem.* 18: 417. 1933.
6. LEIFERT, T., AND O. WATZLAWEK: *Ztschr. physiol. Chem.* 226: 108. 1934.
7. PALMER, J. W., AND H. T. CLARKE: *J. Biol. Chem.* 99: 435. 1933.
8. WEBSTER, B., AND A. M. CHESNEY: *Bull. J. Hopk. Hosp.* 43: 291. 1928.

INFLUENCE OF THYROIDECTOMY ON BLOOD LIPIDS OF THE DOG[1]

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ALTHOUGH an inverse relation between the level of thyroid activity and the content of one or other lipid constituent in the blood of both man and animals has been repeatedly observed (1–5), nothing is known of the mechanism that governs this relation. In an attempt to determine its nature, it seemed desirable to examine first the permanency and degree of consistency of lipid changes that follow complete excision of the gland. In the present investigation, the choleseterol, phospolipids and total fatty acids of the blood were observed at short intervals for as long as 214 days after thyroidectomy. It is shown that the blood lipid response to thyroidectomy is quite irregular and variable. Despite the presence of greatly increased amounts of lipids in the blood of the thyroidectomized dog, sudden reductions in their amounts or even a return to normal concentrations may occur. An unstable blood lipid level appears to be a characteristic feature of the thyroidectomized dog.

EXPERIMENTAL

Excision of the thyroid glands was performed under ether anesthesia. In all cases one or two of the parathyroids were preserved intact with their blood supply. All other parathyroid tissue found was transplanted into neighboring muscle.

The caloric intake of all dogs except *T-8* was maintained constant throughout the period of observation recorded, both before and after excision of the gland. The animals were fed twice daily a diet of whole ground fish and sucrose. The amounts fed each dog are recorded in tables 1 to 3. In addition each dog received 5 gm. of bone ash daily and vitamin supplements twice weekly. Vitamins A and D were supplied in the form of cod liver oil;[2] the B complex as Galen B (Vitab, type II liquid[3]).

The levels of cholesterol, both free and esterified, phospholipid and total fatty acid of the blood of 10 dogs were observed before and after thyroidectomy. Before the operation, the animals were maintained on the standardized diet for 7 to 48 days, during which time several determinations of the blood lipids were made to establish the control or normal levels. During the interval following thyroidectomy, the blood lipids were examined at short intervals; the total periods of observation varied from 4.4 to 9 months. Thirteen to 29 determinations of each lipid constituent were made in each dog.

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[2] The standardized cod liver oil was kindly furnished by Mead Johnson & Company.

[3] The vitamin B concentrate was generously supplied by Vitab Products, Inc., Emeryville, California.

TABLE 1. EFFECTS OF THYROIDECTOMY ON WHOLE BLOOD LIPIDS—EARLY AND MAINTAINED RESPONSE

(All lipid values are expressed as mg. per 100 cc. of whole blood)

Dog No.	Weight	Period of observation				Cholesterol			Total fatty acids	Phospholipids	Total lipids
		Period after thyroidectomy	Diet per day[1]			Total	Free	Ester			
			Days on diet	Fish	Sucrose						
	kg.	days		gm.	gm.						
T-7	15.5	(18)[2]	0	400	60	138	121	17	300	336	438
	15.5	(15)[2]	3	400	60	141	111	30	309	356	450
	15.5	(0)[2]	18	400	60	173	140	33	347	368	520
	16.8	9	27	400	60	226	153	73	392	376	618
	16.1	15	42	400	60	238	135	103	509	420	747
	16.8	36	54	400	60	235	155	80	438	371	673
	17.2	51	69	400	60	242	157	85	519	418	761
	17.2	61	79	400	60	240	139	101	533	394	773
	17.0	74	92	400	60	186	—	—	485	402	671
	17.2	88	106	400	60	219	125	94	483	396	702
	17.1	120	138	400	60	197	120	77	467	405	664
	17.0	121	139	400	60	216	134	82	480	413	696
	17.1	133	151	400	60	250	146	104	506	445	756
T-10	9.5	(32)[2]	7	400	60	173	122	51	385	293	558
	9.5	(27)[2]	12	400	60	161	124	37	379	298	540
	9.6	(0)[2]	39	400	60	198	128	70	380	320	578
	10.0	3	42	400	60	192	152	40	396	347	588
	10.0	5	44	400	60	192	162	30	444	351	636
	10.2	7	46	400	60	233	162	71	460	—	693
	10.5	14	53	400	60	244	157	87	436	—	680
	11.0	43	82	400	60	235	159	76	566	302	801
	11.5	75	114	400	60	292	175	117	505	—	797
	11.6	78	117	400	60	277	184	93	500	—	777
T-12	13.3	(7)[2]	7	400	60	163	135	28	403	316	566
	13.5	(6)[2]	8	400	60	164	140	24	337	358	501
	13.5	(3)[2]	11	400	60	139	125	14	383	307	522
	13.5	(0)[2]	14	400	60	142	125	17	379	308	521
	13.2	5	19	400	60	118	86	32	214	174	332
	14.2	15	29	400	60	311	195	116	471	315	782
	13.6	43	57	400	60	258	190	68	435	362	693
	15.2	78	92	400	60	293	189	104	499	400	792
T-11	18.1	(37)[2]	7	400	60	178	136	42	352	346	530
	18.2	(35)[2]	9	400	60	170	127	43	358	335	528
	18.3	(34)[2]	10	400	60	167	120	47	353	284	520
	18.7	(30)[2]	14	400	60	156	115	41	336	313	492
	18.5	(27)[2]	17	400	60	172	121	51	306	317	478
	18.5	(0)[2]	44	400	60	163	125	38	307	235	460
	18.8	3	47	400	60	159	115	44	364	250	523
	19.0	5	49	400	60	172	126	46	359	335	531
	19.0	7	51	400	60	186	131	55	397	331	583
	19.8	15	59	400	60	261	183	78	663	—	924
	20.5	20	64	400	60	257	150	107	478	287	735
	22.0	56	100	400	60	281	176	105	478	—	759

[1] The amounts recorded were fed in two equal portions at 8:00 A.M. and 4:00 P.M. daily.
[2] The number in parentheses refers to the number of days before thyroidectomy.

Whole blood was used for lipid analyses; the oxidative methods employed have been previously noted (6). The animals were fed twice daily at 8:00 A.M. and at 4:00 P.M. Blood for analyses was taken just before the morning meal was ingested. Thus, at the time blood was sampled, all dogs were in the postabsorptive state, having ingested their last meal approximately 16 hours before removal of blood.

For convenience, the lipid changes observed in the 10 dogs have been classified as 'early and maintained response' (table 1); 'early and fluctuating response' (table 2), and 'poor or no response' (table 3). Although in most cases blood lipid examinations were made at short intervals, it should be noted that more frequent determinations might have altered the classification of an animal's response.

Cholesterol

Before thyroidectomy, determinations for blood cholesterol were made as often as 6 times at short intervals. Under the standardized conditions of the present investigation, the control levels of cholesterol did not show undue fluctuations. Thus in the 5 determinations made in *dog T-14* (table 2) during the 48 days before thyroidectomy, the concentration of total cholesterol in the blood fluctuated from 150 to 155 mg. per 100 cc. of whole blood. This constancy was reflected in both the esterified and the free cholesterol. In the observations on *dog T-15* (table 2) the values for total cholesterol fluctuated from 180 to 192 in 16 days. In 5 of the dogs the control levels did not show the same degree of constancy observed in *T-14* and *T-15*, but even in these (*T-7*, *T-10*, *T-11*, *T-12* and *T-13*) the deviations between the highest and lowest values for total cholesterol did not exceed 37 mg. The highest control values for total cholesterol in the 10 dogs ranged from 135 to 198 mg. per 100 cc. of whole blood.

In 8 of the 10 dogs investigated, striking rises in total cholesterol followed thyroidectomy. The highest value after excision of the gland was found in *T-15*, in which total cholesterol rose to 435 mg., whereas in *T-9* (table 2), *T-12* (table 1) and *T-14* (table 2) the maximum values were 366, 347 and 332 mg. respectively. In these 8 dogs, the maximum values observed after excision of the glands represented increases of 77 to 243 mg. above their corresponding highest control values.

Both fractions of cholesterol were determined, the free and the esterified, and it is of interest to compare the responses of each to thyroidectomy. As already noted the most pronounced rise in total cholesterol occurred in *T-15* (table 2), in which dog free cholesterol rose from a preoperative level of 156 to 209 and esterified from 49 to 226. The latter represents an increase of 362%. In several dogs, values for total cholesterol well over 300 mg. were observed (*T-14*, *T-12* and *T-9*). The most significant responses in these dogs occurred in the esterified portion. Thus in *T-9* (table 2) free cholesterol rose from 125 to 160 mg. while the esterified fraction rose from 44 to 206 mg., an increase of 370%. In *T-12* (table 1) a rise in free cholesterol from 140 to 195 was accompanied by an increase of 315% in esterified cholesterol, viz. from a preoperative level of 24 to a maximum of 116 mg. after thyroidectomy. In *T-14* (table 2) a rise in free cholesterol from 119 to 152 occurred at the same time that the esterified fraction rose from 36 to 170 mg. Not only was the rise in ester cholesterol relatively more significant, but its occurrence was also more regular. Cases in which rises in ester cholesterol were found associated with no response in the free portion were not uncommon. In dogs in which total cholesterol did not rise above 300 mg., the changes were reflected mainly in the esterified portion. In *T-14* (table 2) total cholesterol rose to 300 mg. 146 days after thyroidectomy; this represented a rise of 145 mg. above the preoperative level and, with the exception of 18 mg., was due entirely to the esterified portion. In *T-7* (table 1) the entire response in cholesterol was due to its combined fraction. Blood was examined 24 times in *T-14* during a period of 214 days after thyroidectomy. On only 2 occasions were increases in free cholesterol of about 40 mg. observed, whereas the esterified portion repeatedly and frequently showed increases of 200 to 425%.

The time of onset of cholesterol changes after thyroidectomy is of interest. Lipid examinations were made as early as 3 days (*T-10* and *T-11*) after excision of both

TABLE 2. THE EFFECTS OF THYROIDECTOMY ON WHOLE BLOOD LIPIDS—EARLY AND FLUCTUATING RESPONSE

(All lipid values are expressed as mg. per 100 cc. of whole blood)

Dog No.	Weight	Period of observation				Cholesterol			Total fatty acids	Phospholipids	Total lipids
		Period after thyroidectomy	Diet per Day[1]			Total	Free	Ester			
			Days on diet	Fish	Sucrose						
	kg.	*days*		*gm.*	*gm.*						
T-9	11.0	(35)[2]	18	300	60	162	122	40	388	388	550
	11.0	(12)[2]	41	300	60	167	123	44	373	375	540
	11.0	(7)[2]	46	300	60	169	125	44	375	372	544
	11.0	(0)[2]	53	300	60	162	122	40	379	380	541
	10.9	8	61	300	60	207	133	74	460	403	667
	11.1	10	63	300	60	212	141	71	474	423	686
	11.2	15	68	300	60	246	130	116	568	450	814
	11.5	25	78	300	60	292	155	137	548	407	840
	11.5	32	85	300	60	259	143	116	476	—	735
	11.6	37	90	300	60	281	157	124	598	378	879
	11.6	46	99	300	60	230	141	89	450	392	680
	11.5	53	106	300	60	280	145	135	525	416	805
	11.5	60	113	300	60	245	137	108	430	472	675
	11.5	74	128	300	60	274	143	131	579	438	853
	11.6	88	141	300	60	257	127	130	636	456	893
	11.5	103	156	300	60	269	142	127	626	407	895
	11.7	118	161	300	60	270	157	113	582	378	852
	11.5	129	182	300	60	292	156	136	664	427	956
	11.6	145	198	300	60	366	160	206	712	493	1078
	11.5	151	204	300	60	349	149	200	585	488	934
	11.6	160	213	300	60	361	164	197	716	495	1077
	12.0	180	233	300	60	267	143	124	530	—	797
	12.0	209	262	300	60	316	142	174	468	426	784
	12.0	213	269	300	60	284	139	145	510	447	794
T-15	7.9	(16)[2]	48	300	100	186	137	49	381	363	567
	7.6	(3)[2]	61	300	100	192	156	36	365	366	557
	7.8	(0)[2]	64	300	100	180	130	50	380	342	560
	7.5	7	71	300	100	181	130	51	383	338	564
	8.4	29	93	300	100	271	160	111	465	412	736
	8.8	41	103	300	100	318	143	175	564	405	882
	10.0	68	132	300	100	346	175	171	618	437	964
	11.0	91	155	300	100	435	209	226	857	529	1292
	10.9	100	164	300	100	422	209	213	676	—	1098
	11.0	101	165	300	100	306	174	132	678	—	984
	11.0	102	166	300	100	402	201	201	725	334	1127
	11.1	107	171	300	100	368	200	168	728	—	1096
	11.4	113	177	300	100	380	191	189	761	488	1141
T-14	10.0	(48)[2]	5	300	60	155	119	36	310	352	465
	10.0	(28)[2]	25	300	60	150	117	33	345	358	495
	10.0	(7)[2]	46	300	60	153	118	35	338	350	491
	10.4	(5)[2]	48	300	60	153	120	33	369	347	522
	10.0	(0)[2]	53	300	60	153	120	33	347	369	500
	10.0	11	64	300	60	158	127	31	352	328	510
	10.1	16	69	300	60	196	114	82	416	338	612
	10.1	26	79	300	60	250	136	114	440	368	690
	10.4	33	86	300	60	264	155	109	522	342	786
	10.6	40	93	300	60	323	149	174	592	351	915
	10.8	47	100	300	60	265	155	110	490	300	755
	10.8	54	109	300	60	259	164	95	471	379	730
	11.1	61	114	300	60	209	130	79	437	350	646
		66	119	300	60	153	118	35	350	338	503
	11.1	68	121	300	60	232	148	84	417	349	649
	11.3	75	128	300	60	248	163	85	459	335	707
	11.5	89	142	300	60	226	146	80	387	448	613
	11.5	98	151	300	60	228	139	89	435	382	663

TABLE 2—*Continued*

Dog No.	Weight	Period of observation				Cholesterol			Total fatty acids	Phospholipids	Total lipids
		Period after thyroidectomy	Diet per Day[1]								
			Days on diet	Fish	Sucrose	Total	Free	Ester			
	kg.	*days*		*gm.*	*gm.*						
	11.5	103	156	300	60	226	146	80	448	387	674
	11.6	104	157	300	60	218	126	92	447	323	665
	11.6	119	172	300	60	332	152	180	607	417	939
	11.5	130	183	300	60	249	130	119	477	333	726
	11.7	146	199	300	60	300	138	162	493	323	793
	11.2	152	205	300	60	226	127	99	400	310	626
	11.2	161	214	300	60	205	139	66	421	322	626
	10.8	177	230	300	60	216	120	96	449	444	665
	11.4	180	233	300	60	246	123	123	422	366	668
	11.3	210	263	300	60	266	134	132	350	318	616
	11.3	214	267	300	60	300	137	163	464	363	764
Br.F.	14.0	(38)[2]	19	400	20	135	108	27	328	306	463
	14.0	(0)[2]	57	400	20	141	116	25	337	351	478
	14.4	7	64	400	20	219	132	87	501	363	720
	15.0	16	73	400	20	187	152	35	374	366	561
	13.4	27	84	400	20	123	101	22	272	190	395
	15.7	31	88	400	20	205	159	46	510	356	715
	16.5	38	95	400	20	220	107	113	452	385	672
	15.0	54	111	400	20	161	125	36	333	323	494
	15.0	68	125	400	20	140	122	18	404	353	544
	15.5	93	150	400	20	149	111	38	396	326	545
	15.0	117	174	400	20	236	153	83	379	328	615
	14.4	129	186	400	20	188	111	77	404	409	592
	14.7	135	192	400	20	183	110	73	437	360	620

[1,2] See table 1.

glands, but the earliest rise was observed after 7 days (*T-10; Br. F*). In most cases this initial rise made its appearance abruptly by increases of 30 to 70 mg. The blood of *T-11* was examined on the 3rd, 5th, 7th and 15th day after thyroidectomy. On the first 3 occasions no change in total cholesterol occurred. In the interval between 7 and 15 days, the blood cholesterol had increased by 75 mg. The most striking case of an abrupt rise occurred in *T-12*, in which in the interval from 5 to 15 days after thyroidectomy the concentration of total cholesterol in the blood rose from 118 to 311 mg.

The time of appearance of maximum levels of cholesterol in the blood after thyroidectomy is quite variable. In *Br. F.* and *T-7*, values close to the maximum, namely 219 and 226 mg., were found as early as 7 and 9 days respectively after thyroidectomy. In *T-12* the maximum was observed 15 days after thyroidectomy. In the other animals there was considerable delay, in *T-9* as long as 145 days, before maximum concentrations appeared.

Fluctuations in the cholesterol levels were quite common. Moreover, no regularity in the types of fluctuations was observed in the 8 dogs whose lipids responded to thyroidectomy. In some dogs (table 1) high cholesterol values appeared early after thyroidectomy, and the elevated level observed during the rest of the long period of study was characterized by only slight fluctuations. In others (table 2) early rises in cholesterol followed excision of both thyroids, but as a rule this was interrupted at one or more points by a marked fall and a subsequent return to the elevated level. Just as elevated levels made their appearance abruptly, so also losses of cholesterol from the blood occurred with amazing suddenness. A good example of this occurred in dog *T-15* (table 2). On the 91st and 100th day after thyroidectomy, the blood of this

TABLE 3. EFFECTS OF THYROIDECTOMY ON WHOLE BLOOD LIPIDS—POOR OR NO RESPONSE

(All lipid values are expressed as mg. per 100 cc. of whole blood)

Dog No.	Weight	Period of observation				Cholesterol			Total fatty acids	Phospholipids	Total lipids
		Period after thyroidectomy	Diet per day[1]			Total	Free	Ester			
			Days on diet	Fish	Sucrose						
	kg.	days		gm.	gm.						
T-8	16.0	(14)[2]	14	400	20	157	124	33	292	313	449
	15.7	(3)[2]	25	400	20	157	122	35	362	339	519
	15.4	(0)[2]	28	400	20	155	120	35	372	350	527
	15.0	9	37	400	20	194	128	66	280	304	474
	15.1	15	43	400	20	191	134	57	358	321	549
	14.0	36	64	400	20	167	120	47	328	286	495
	13.5	51	79	400	20	154	121	33	359	289	513
	13.2	56	84	400	20	174	115	59	312	298	486
	13.2	58	86	400	20	161	100	61	362	287	523
	13.5	61	89	400	20	163	100	63	364	299	527
	13.5	62	1	800	40	165	100	65	364	299	529
	14.1	66	5	800	40	164	108	56	370	389	534
	15.0	68	7	800	40	151	100	51	324	321	475
	15.0	74	13	800	40	151	—	—	324	321	475
	16.5	88	27	800	40	122	95	27	355	311	477
	18.0	111	50	800	40	138	99	39	319	327	457
	18.4	122	61	800	40	152	116	36	360	328	512
	18.0	123	62	800	40	148	114	34	328	292	476
	18.0	124	63	800	40	140	102	38	360	257	500
	19.0	139	78	800	40	126	95	31	345	294	471
T-13	9.0	(26)[2]	7	400	60	179	127	52	420	388	599
	9.9	(20)[2]	13	400	60	181	115	66	424	340	605
	9.8	(17)[2]	16	400	60	169	120	49	447	330	616
	10.1	(0)[2]	33	400	60	179	126	53	417	396	596
	10.1	9	42	400	60	201	129	72	464	412	665
	10.5	20	53	400	60	186	138	48	481	377	667
	10.5	26	59	400	60	184	143	41	410	457	594
	10.5	35	68	400	60	179	131	48	449	476	628
	10.5	41	74	400	60	186	120	66	422	404	608
	10.1	50	83	400	60	179	126	53	316	417	495
	11.0	55	88	400	60	180	129	51	448	373	628
	10.8	67	100	400	60	180	135	45	379	383	559
	10.5	84	117	400	60	186	138	48	377	481	563
	10.4	89	122	400	60	181	127	54	440	343	621
	10.5	99	132	400	60	181	120	61	441	364	622
	10.8	115	148	400	60	216	120	96	444	449	660
		150	183	400	60	212	134	78	419	394	631
		154	187	400	60	204	112	92	492	400	696

[1,2] See table 1.

dog contained 435 and 422 mg. of total cholesterol, respectively. On the 101st day, i.e. 24 hours after the latter observation had been made, the value found for total cholesterol was 306 mg. On the 102nd day, 402 mg. of this lipid constituent were contained in each 100 cc. of whole blood. The fluctuations in T-14 are also of interest, because on the 66th day after thyroidectomy the concentration of total cholesterol in its blood had dropped to that found during the preoperative period despite the fact that elevated levels (209–323) had been maintained from the 26th to 61st day after thyroidectomy. Fluctuations in the cholesterol levels of the blood have also been observed in the thyroidectomized dog by Schmidt and Hughes (3) and in the thyroidectomized rabbit by Fleischmann et al. (5).

In 2 dogs, singular responses of blood cholestrol to thyroidectomy were obtained (table 3). In T-8, on only 2 occasions were cholesterol values significantly above pre-

operative levels found during the first 61 days after thyroidectomy. This dog was observed for 139 days after thyroidectomy, and during the last 78 days its caloric intake was doubled. Despite a gain of over 5 kg. during this latter period, no measurable increase in the cholesterol levels of the blood was obtained. T-13 (table 3) was studied for 154 days after thyroidectomy. A noticeable rise in the cholesterol level of this animal did not appear until 115 days had elapsed after thyroidectomy; the elevated level of cholesterol once established, however, remained apparently throughout the rest of the period of observation (39 days).

Phospholipids

In 5 of the dogs, the levels of phospholipid found during the control period showed little fluctuation. Thus T-9 was examined on 5 occasions during an interval of 37 days before thyroidectomy; during this time the maximum and minimum values observed for phospholipid were 450 and 378 mg. The preoperative variations in the blood of T-7, T-8, T-10 and T-14 ranged from 22 to 37 mg.; in T-12, 50 mg. and in *Br. F.*, 45 mg. The control levels of phospholipids in 2 dogs, T-11 and T-13, showed considerable instability; the differences between maximum and minimum values in these animals were respectively 111 and 66 mg.

No changes in phospholipids as striking as those observed in esterified cholesterol or total fatty acids were obtained. In cholesterol esters, elevations of 300% above the control levels were not uncommon, whereas in only a single dog (T-15) did the maximum response in phospholipids represent a gain of as much as 45% above the highest preoperative value. Even this rise of 45% in T-15 was observed at a single interval after thyroidectomy, namely 91 days; all other values observed after excision of the glands represented gains of not more than 33% above the control level. The maximum values found before and after thyroidectomy in T-7 and T-9 were respectively 368 and 445, and 388 and 495. These elevations represent increases of 21 and 28% respectively but in these animals the elevated phospholipid levels were well maintained throughout the 133 and 213 days after thyroidectomy. T-14 was observed for 214 days after thyroidectomy, during which time the blood phospholipids were determined on 24 separate occasions. The maximum values observed before and after thyroidectomy were respectively 369 and 448 mg. per 100 cc. of whole blood; the latter represents an increase of 22% above the former. But it should be noted that on only 3 occasions after thyroidectomy were the values found as high as 22% above the control values. On 21 out of 24 occasions the phospholipid values in this dog were either below, or not significantly above, the highest control values. In T-10, T-12, T-13 and *Br. F.*, changes in the level of phospholipid that followed thyroidectomy did not exceed 12% and hence should not be considered a significant rise.

Total Fatty Acids

The total fatty acid content of the blood of all dogs during the control period showed a good degree of stability. All preoperative values in T-10 and *Br. F.* were in excellent agreement; in these the differences between maximum and minimum values did not exceed 9 mg. The variations in T-9, T-13 and T-15 amounted to 15, 30 and 16 mg. respectively. The agreement among the control values in T-14, T-12, T-11 and T-7 was not as good as that observed in the dogs recorded above; yet the fluctuations in these dogs were 59, 66, 52 and 47 mg. respectively. The greatest irregularity was found in T-8, in which on the day of thyroidectomy and 3 days before this the total fatty acid levels were 372 and 362 mg. respectively, but 14 days before operation this level was 292 mg.

Pronounced responses of total fatty acids to thyroidectomy were obtained in 7 of the 10 dogs examined. The highest value for total fatty acids was found in *dog T-15*, in which this lipid constituent rose from a preoperative level of 365–381 to 857 mg., an increase of 125%. The maximum values in *T-9* and *T-11* represented increases of 85 and 88% respectively above control values. Lesser though significant responses were obtained in *T-7*, *T-10*, *T-14* and *Br. F.*; in these the maximum values were 47 to 65% above preoperative levels. In 2 dogs (*T-8* and *T-13*) none of the values for total fatty acids found after thyroidectomy were significantly above those for the previously established control period. These dogs were observed for 139 and 154 days respectively after thyroidectomy. In the former, doubling the caloric intake during the last 78 days failed to alter the total fatty acid content of the blood.

Elevated levels of total fatty acids were present in all of the 7 dogs that responded by the time 29 days had elapsed after thyroidectomy. A significant rise in total fatty acids may occur, however, as early as one week after excision of both glands. On the 5th day after operation the concentration of this lipid constituent in *T-10* had risen from a preoperative level of 379–385 to 444 mg.; in 7 days total fatty acids in *Br. F.* rose from 337 to 501 mg. In *T-9*, *T-11*, *T-14* and *T-15* initial responses in total fatty acids were found on the 8th, 15th, 16th and 29th day following thyroidectomy. The most spectacular of the earliest responses occurred in *T-11*, in which thyroidectomy produced an increase from 306–358 to 663, a gain of 88% above the preoperative levels.

As already noted, 3 dogs failed to show significant rises in total fatty acids after thyroidectomy. But even in the 7 dogs in which definite increases did occur, no uniform type of response was observed. In *T-7* an elevated level was noted early and maintained throughout the period of observation (133 days after thyroidectomy). Somewhat similar types of response were obtained in *T-10* and *T-12*. *T-15* was observed for 113 days after thyroidectomy. During the first 91 days there was a steady climb in the total fatty acid level. Ten days later the concentration of this lipid constituent decreased by 181 mg. and, although total fatty acid levels during the subsequent 12 days were well above those observed during the preoperative period, nevertheless they never rose to the high value found on the 91st day.

In 3 dogs (*T-14*, *T-11* and *T-9*) fluctuating levels of total fatty acids were observed after thyroidectomy. The most pronounced case of this was found in *T-14*. In this dog, total fatty acids rose steadily during the first 40 days after the operation, then steeply declined to a point where the concentration on the 66th day after thyroidectomy was no higher than that found before the operation. Thereafter marked fluctuations continued and, although a value of 607 mg. was found on the 119th day, values close to the preoperative were also observed on 2 other occasions, namely 89 and 210 days after thyroidectomy.

Caloric intake and weight changes after thyroidectomy. As shown in tables 1 to 3, the caloric intake per kg. of body weight was not equal in the 10 dogs. In 9 of them, however, the caloric intake was kept constant throughout the period of observation, i.e. during the preoperative and postoperative periods. The weights of all dogs showed practically no change during the 1 to 7 weeks before thyroidectomy in which control measurements were made. An increase in weight, but one variable in degree, was found in all dogs after thyroidectomy. Thus *T-7*, *T-10*, *T-12* and *T-11*, whose preoperative weights were respectively 15.5, 9.6, 13.5 and 18.5 kg., gained approximately 2 kg. 78 days after thyroidectomy. It was these animals that showed an early and well maintained rise in blood lipids after thyroidectomy. Weight increases were also observed in *dogs T-14* and *T-15* (table 2); their blood lipids also responded well to thyroidectomy. It should not be inferred from these observations, however, that a

gain in weight is necessarily associated with a rise in blood lipids. Thus in T-14 total lipids rose from preoperative values of 465–500 mg. to 915 mg. 40 days after thyroidectomy; at the latter interval no significant increase in weight had occurred.

SUMMARY

In the present investigation, an examination was made of the response of whole blood lipids to excision of thyroid tissue in the dog. The levels of blood cholesterol both free and esterified, phospholipids and total fatty acids were compared in 10 dogs before and after thyroidectomy. The caloric intake in 9 of these dogs was maintained constant throughout the period of observation, both before and after thyroidectomy. Lipid determinations of the blood were made at short intervals for as long as 214 days after thyroidectomy. From the data presented, the characteristic response of each lipid constituent may be pointed out.

Although lipid levels of the blood rose after thyroidectomy, the various lipid constituents did not respond to the same degree. The most pronounced increases occurred in cholesterol and in total fatty acids.

Striking changes in cholesterol esters occurred after thyroidectomy. The highest values observed after thyroidectomy represented increases of 37–380% above the highest preoperative values; in 4 cases the increases were over 300%.

Changes in the levels of cholesterol esters may appear early after thyroidectomy. An 87% increase in esterified cholesterol was observed as early as 7 days after excision of the thyroid glands, and in 7 of the dogs significant changes in the levels of this lipid constituent were present when 16 days had elapsed after thyroidectomy.

Increases in the total fatty acid content of the blood occurred in 7 of the 10 dogs after thyroidectomy. In no case did the extent of the rise in total fatty acids approach that recorded for esterified cholesterol. In a single animal the maximum increase in total fatty acids represented an increase of 125% above the preoperative value. In the 6 other dogs that responded, the maximum values after thyroidectomy were 47 to 88% above the preoperative.

A significant rise in total fatty acids may occur as early as one week after thyroidectomy. In 7 of the 10 dogs examined, definite changes were present by the time 29 days had elapsed.

Pronounced changes in the levels of free cholesterol or of phospholipids were observed infrequently after thyroidectomy.

The types of response of the blood lipids to thyroidectomy differed widely. The responses among the 10 dogs examined were not uniform as regards either the extent of the rise, or the time of onset of lipid increases, or the maintenance of elevated levels, or the degree of fluctuations. Rapidly changing levels of esterified cholesterol and total fatty acids were observed in a number of the dogs after removal of the thyroid glands.

REFERENCES

1. Boyd, E. M.: *Trans. Royal Soc. Canada* 30: 11. 1936. Contains a review of earlier work.
2. Boyd, E. M., and W. F. Connell: *Quart. J. Med.* 5: 455. 1936.
3. Schmidt, L. H., and H. B. Hughes: *Endocrinology* 22: 474. 1938.
4. Gildea, E. F., E. B. Man and J. P. Peters: *J. Clin. Investigation* 18: 739. 1939.
5. Fleischmann, W. H. B. Shumacker, Jr., and L. Wilkins: *Am. J. Physiol.*, 131: 317. 1940.
6. Chaikoff, I. L., and A. Kaplan: *J. Biol. Chem.* 106: 267. 1934.

NITROGEN METABOLISM IN HYPERTHYROIDISM[1]

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THAT creatinuria accompanies hyperthyroidism in mammals has been known for years (1). In 1936 Bodansky and Duff (2) demonstrated that the administration of thyroxin or desiccated thyroid to rats is followed by increased nitrogen and creatine excretion, the effect being relatively very small at very high temperatures (33–34°C.) and becoming more marked at ordinary room temperatures. That the augmented creatine metabolism depended on the presence of thyroid seemed probable from the fact that it did not occur in thyroparathyroidectomized rats. Recently, Allison, Glaser, and Leonard (3) reported that thyroidectomy decreases creatine excretion and stated that this decrease always may be accompanied or followed by an increase in creatinine. Glycogen depletion in hyperthyroidism (4) may be a factor in creatinuria just as it is in starvation. That the changes in the muscle, both anatomical and metabolic, occurring in hyperthyroidism resemble those in progressive muscular dystrophy has been pointed out by Shorr and associates (5). The observations of Bodansky (6) that the injection of thyroxin in the rat results in a reduction of 20 to 30% in muscle creatine and in 40 to 50% in heart creatine have been repeatedly confirmed in this laboratory. We have, however, found that accurate results can be obtained only on the dry basis because of the great variations frequently found in the water content of both heart and muscle. There is also evidence that there is a 25 to 50% reduction in adenyl pyrophosphoric acid in the hyperthyroid heart (7). Such reduction is claimed to be prevented by the administration of vitamin C.

The claim of Fischer and Oehme (8) that 40 mg. of ascorbic acid daily to rats suppressed the creatinuria induced by thyrotoxicosis we have been unable to confirm after repeated trials (9). The claim of David (10) that massive doses of Vogan (vitamin A) will prevent creatine losses in the myocardium in thyroxinized rats we have been also unable to substantiate (9), using 30,000 to 60,000 I.U. daily for a period of 13 days, on a daily dose of 0.7 mg. synthetic thyroxin.

Our work in progress (9) indicates that it may be possible to secure considerable protection against losses of muscle creatine in thyroxinized rats on a high fat diet with the following daily doses of the various components of the vitamin B complex: 100 γ thiamin, 100 γ riboflavin, 50 γ pyridoxin, and 1 cc. of the "W" factor, equivalent to 1 gm. liver extracts. The preventive influence of such diet against losses of muscle creatine we anticipate is probably due to the sparing action of fats on protein catabolism. While this work is being continued in attempts to prevent losses of creatine in the myocardium, we thought it would be of interest to determine the influence

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of hyperthyroidism on the distribution of the non-protein nitrogen of the blood and on the partition of nitrogen in the urine. Such results are submitted in tables 1 to 3 inclusive.

METHODS OF STUDY

Simple and accurate available microchemical methods for the measurement of the non-protein nitrogen constituents of the blood require more blood than can be obtained from the living rat. The spectrophotometer methods of Borsook (11) and the electro titration methods of Borsook and Dubnoff (12) were considered too involved for routine use. With the aid of the Evelyn macro-micro photoelectric colorimeter (13), a technic was developed for micro determinations of non-protein nitrogen, urea nitrogen, creatine and creatinine. The details of the procedures appeared elsewhere (14).

One cc. of caudal blood was found sufficient for all the determinations of the non-protein constituents of the blood. Since in all types of animal experimentation considerable individual variations are encountered in determinations of blood constituents, it was decided to pool the blood from several groups of animals subjected to the same treatment and showing similar biological symptomatology. Enough blood can be secured by caudal bleeding from 3 animals weighing 60 to 70 gm. Such technic has made it possible for the first time to follow the nitrogen partition of the blood of the living small animal and we are proceeding successfully in obtaining the blood picture during the development of various stages of the deficiency diseases.

For the partition of the nitrogen of the urine we have used standard methods with slight modifications. The collection of urine was made as follows. The animals are placed in round metabolism cages set on large funnels. In the center of the funnel a layer of 5/16″ mesh galvanized wire of 26-gauge collects the feces and in the bottom of the funnel a small layer of the same wire covered with filter paper, moistened with N/10 HCl, collects any small particles of food that may spill. The funnels are set on large wooden filters, each of which accommodates 6 metabolism cages. These filters we found much firmer supports for the funnels than the customary tripods used. Each week was divided into 3 metabolism periods, 2 of 48 hours, and one of 72 hours duration. The urine was collected into half-pint Mason jars in which were introduced 20 cc. of N/10 HCl and 30 drops of toluol. At the end of the day the funnels were washed with a fine spray of about 15 cc. of distilled water. In the morning the animals and feed were removed for weighing during which time the funnels were washed thoroughly with distilled water. The cages were left on the funnels and the washing was done from the wire bottoms of the cages, since dried urine may have been left there. The urine washings were collected in one quart Mason jars and stored in the refrigerator. At the end of the metabolism period the urine of each animal was diluted to 500 cc. If pooled urines were used from several animals corresponding larger dilutions were made.

For the total nitrogen determination 100 cc. aliquots of the urine washings were taken. After digestion, dilution was made to 500 cc. and 20 cc. aliquots were used for analysis by steam distillation at atmospheric pressure on a micro scale.

The ammonia and urea determinations were carried out by the Van Slyke and Cullen aeration method (15). One cc. of a 10% solution of Squibb's urease and 6 gm. of powdered sodium carbonate were used for each determination. For the urea 5 cc. and for the ammonia 10 cc. of the diluted urine were taken.

For the uric acid determination 0.5 cc. samples were used and the readings were taken in the Evelyn macro colorimeter with a 660 filter. For the preformed and total creatinine 5 cc. samples of the diluted urine were taken and the determinations were carried out according to Folin (16), using the 520 filter in the Evelyn macro instru-

TABLE 1. INFLUENCE OF SUBCUTANEOUS INJECTIONS OF THYROXIN ON PARTITION OF NITROGEN IN URINE OF THE ALBINO RAT

T. N. = total nitrogen.

(Average results of Series I experiments)

Started to inject 0.5 mg. synthetic thyroxin to each A animal daily 8/5; increased to 1.0 mg. daily 8/10.

Date 1940	Groups	Metabolism period	Changes in weight#	Total N	Urea N		Ammonia N		Allantoin		Uric acid		Preformed creatinine		Creatine	
					N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.
		days	*gm.*	*mg.*	*mg.*		*mg.*		*mg.*		*mg.*		*mg.*		*mg.*	
7/24	3—A*	2		1351.4	1002.1	74.1	116.4	8.6	171.1	12.7	10.8	0.79	15.9	1.18	6.7	0.51
	B*			1301.8	1049.7	80.7	99.9	7.6	141.8	10.9	11.0	0.84	13.7	1.05	10.2	0.77
7/26	3—A	2	+11	1315.0	871.7	66.3	92.4	7.0	194.7	14.8	11.3	0.86	18.4	1.40	0.67	0.05
	B		+14	1381.7	930.3	67.4	95.9	6.9	140.9	10.2	11.3	0.82	20.8	1.50	0.70	0.05
7/29	3—A	3	+ 7	2314.2	1868.7	81.0	115.6	5.0	106.8	4.6	16.9	0.73	28.5	1.23	4.3	0.18
	B		+13	2270.7	1806.2	84.4	107.5	4.7	116.7	5.1	16.9	0.74	31.1	1.37	5.1	0.22
7/31	3—A	2	+14	1506.6	1205.9	80.0	62.6	4.1	84.3	5.6	8.4	0.56	18.7	1.24	1.6	0.11
	B		+ 6	1535.4	1198.7	78.1	62.6	4.1	116.2	7.6	8.3	0.54	21.1	1.37	2.1	0.13
8/2	3—A	2	+16	1303.9	1035.1	79.3	68.4	5.2	111.5	8.6	9.1	0.69	10.1	0.77	2.1	0.16
	B		+15	1266.3	1016.5	80.3	52.1	4.1	100.0	7.9	8.7	0.69	11.4	0.90	1.9	0.15
8/5	3—P	3	+20	1978.0	1581.4	80.0	92.3	4.7	115.4	5.8	14.8	0.75	29.9	1.51	4.1	0.21
	RC		+ 9	2091.6	1682.6	80.4	90.8	4.3	165.9	7.9	14.3	0.68	33.3	1.59	3.6	0.17
8/7	3—P	2	−11	1479.1	1202.4	81.4	63.8	4.3	80.3	5.4	10.0	0.67	18.8	1.27	16.0	1.09
	RC		+19	1472.6	1191.4	80.8	49.2	3.3	93.3	6.3	7.6	0.52	24.8	1.69	0.8	0.05
8/9	3—P	2	−25	1604.8	1195.5	74.5	68.4	4.3	168.0	10.5	13.6	0.85	18.2	1.13	32.6	2.03
	RC		+ 2	1536.9	1149.6	74.8	64.4	4.2	185.9	12.1	10.5	0.68	22.6	1.46	6.3	0.41
8/12	3—P	3	−23	2456.1	1767.1	71.1	112.1	4.6	327.9	13.3	18.3	0.79	32.2	1.31	33.3	1.43
	RC		+ 5	2397.2	1797.7	75.0	90.7	3.8	334.5	13.9	14.5	0.60	37.0	1.54	7.6	0.32
8/14	2—P	2	−17	1964.9	1517.0	77.2	89.0	4.5	192.4	9.8	14.7	0.75	22.2	1.13	22.8	1.16
	RC		− 9	1483.1	1192.3	80.4	56.8	3.8	133.8	9.0	9.3	0.63	25.8	1.72	1.4	0.01

8/16	3—P	2	−11	2544.7	2050.1	80.5	128.7	5.0	137.6	5.4	18.4	0.72	22.8	0.89	33.6	1.32
	RC		+27	1748.0	1460.1	83.5	62.7	3.6	83.8	4.8	9.3	0.53	24.7	1.41	6.4	0.04
8/19	3—P	3	−13	4620.1	3639.1	78.8	325.3	7.0	317.0	6.9	36.7	0.79	32.6	0.70	46.2	1.00
	RC		+ 6	3049.2	2503.3	82.1	104.9	3.4	188.2	6.2	15.0	0.49	38.7	1.27	7.7	0.22
8/21	3—P	2	± 0	2975.9	2161.9	72.6	162.9	5.4	431.7	11.2	22.0	0.74	19.9	0.67	18.6	0.63
	RC		+15	2387.1	1957.0	81.9	73.9	3.1	169.3	7.1	10.2	0.42	23.1	0.97	7.1	0.29
8/23	3—P	2	−14	2951.9	2134.3	72.3	166.4	5.6	450.9	15.2	22.4	0.76	16.9	0.57	20.5	0.69
	RC		+32	2487.9	1899.3	76.3	87.7	3.5	352.6	14.1	11.0	0.51	18.3	0.73	7.8	0.31
8/24	1—P§$^{-1}$	1	−19	478.5	396.0	82.7	22.5	4.7	28.3	5.9	4.1	0.86	2.4	0.50	2.0	0.42
	RC^{-1}		− 2	336.0	279.6	83.3	9.4	2.8	33.6	10.0	2.1	0.62	3.7	1.10	1.0	0.29
8/26	2—P†	3	− 8	2638.5	2138.5	81.0	165.8	6.3	232.3	8.8	21.6	0.82	15.8	0.60	18.1	0.68
	RC		+20	1940.4	1619.6	83.5	65.0	3.3	200.9	10.3	6.8	0.35	25.2	1.30	4.1	0.21
8/28	1—P^{-2}	2	− 2	877.4	683.6	78.0	69.7	7.9	72.5	8.2	9.6	1.09	5.9	0.66	7.0	0.79
	RC^{-2}		+ 2	657.2	544.3	82.9	17.0	2.5	54.1	8.2	3.1	0.49	8.9	1.35	1.2	0.18
8/30	1—P^{-3}	2	− 3	954.0	711.8	74.6	82.6	8.7	83.4	8.7	2.9	0.30	5.2	0.55	4.6	0.46
	RC^{-3}		+ 4	653.6	507.6	77.7	22.3	3.4	71.3	10.9	1.4	0.22	7.6	1.19	1.0	0.16
9/2	1—P^{-3}	3	−12	1309.1	1059.7	80.9	121.3	9.3	116.0	8.9	11.1	0.83	8.5	0.65	4.6	0.35
	RC^{-3}		± 0	827.5	707.9	85.5	16.3	2.0	85.0	10.3	4.2	0.50	10.2	1.23	2.3	0.28
9/4	1—P^{-3}	2	+ 2	460.1	351.3	76.3	37.8	8.2	36.6	8.0	5.5	1.19	2.9	0.63	2.3	0.50
	RC^{-3}		+ 2	234.4	176.8	74.8	5.8	2.5	26.4	11.3	1.6	0.68	4.1	1.71	0.2	0.08
9/6	1—P^{-3}	1	−12	386.9	295.2	76.2	46.9	12.1	38.5	9.9	3.7	1.25	2.4	0.81	2.3	0.78
	RC^{-3}		− 1	227.2	182.5	80.3	3.2	1.4	30.3	13.3	1.7	0.75	3.0	1.64	1.1	0.42

* A represents the animals which later received thyroxin and subsequently referred to as P.

* B represents the litter mates of A animals, which were given the same ration and restricted to the same amount of food consumed by the A rats, and later referred to as RC (restricted controls).

§ The P animal of group 1 (marked P^{-1} and RC^{-1}) died 8/25, leaving 2 groups in this series.

† The P animal of group 2 died during night of 8/27, leaving only one group in this series (marked P^{-3} and RC^{-3}).

During metabolism intervals.

TABLE 2. INFLUENCE OF SUBCUTANEOUS INJECTIONS OF THYROXIN ON PARTITION OF NITROGEN IN URINE OF THE ALBINO RAT

T. N. = total nitrogen.
(Average results of Series II experiments)
Started to inject 0.5 mg. synthetic thyroxin to each A animal daily 8/6; increased to 1.0 mg. daily 8/10.

Date 1940	Groups	Metabolism period	Changes in weight#	Total N	Urea N		Ammonia N		Allantoin N		Uric acid		Preformed creatinine		Creatine	
					N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.	N	% of T. N.
		days	gm.	mg.	mg.		mg.		mg.		mg.		mg.		mg.	
7/25	3—A*	2		797.0	567.7	71.3	88.2	11.0	76.3	9.5	8.6	1.08	16.7	2.10	4.3	0.54
	B*			753.5	536.1	71.1	75.9	10.1	74.8	9.9	9.3	1.23	18.7	2.50	4.3	0.57
7/27	3—A	2	+24	789.9	565.2	71.5	52.7	6.7	88.9	11.2	7.6	0.96	9.1	1.20	2.0	0.25
	B		+18	677.7	477.5	70.5	35.1	5.2	86.1	12.7	6.8	1.00	8.5	1.30	1.6	0.23
7/30	3—A	3	+25	1100.1	802.6	73.0	88.5	8.0	124.6	11.3	12.9	1.17	13.2	1.20	2.0	0.18
	B		+18	999.6	722.5	72.2	61.3	6.1	113.0	11.3	11.9	1.19	11.8	1.18	2.8	0.28
8/1	3—A	2	+22	648.4	461.4	71.2	55.8	8.6	87.3	13.5	7.2	1.11	7.5	1.16	1.3	0.20
	B		+21	670.2	490.2	73.1	39.7	5.9	84.0	12.5	6.8	1.01	9.3	1.39	1.8	0.27
8/3	3—A	2	+15	670.1	448.7	67.0	75.7	11.3	89.4	13.3	6.7	1.00	9.8	1.46	2.1	0.31
	B		+18	705.3	510.2	72.3	51.0	7.2	80.5	11.4	7.0	0.99	9.1	1.29	2.0	0.28
8/6	3—P	3	+27	1236.2	883.7	71.5	111.8	9.0	161.3	13.0	12.9	1.04	16.7	1.35	3.1	0.26
	RC		+19	1327.6	983.1	74.0	1064.	8.0	151.1	11.4	10.8	0.81	15.3	1.15	4.0	0.30
8/8	3—P	2	− 4	1013.6	753.0	74.3	88.1	8.7	91.8	9.0	12.6	1.24	13.8	1.36	16.0	1.57
	RC		+29	1056.6	787.5	74.5	88.1	8.3	124.3	11.8	10.2	0.96	12.0	1.13	3.3	0.31
8/10	3—P	2	− 7	1099.6	787.5	71.6	73.7	6.7	124.3	11.3	11.2	1.02	12.0	1.09	16.4	1.50
	RC		+ 7	1045.9	769.6	73.6	73.8	7.0	129.3	12.4	9.1	0.87	12.0	1.14	2.2	0.21
8/13	3—P	3	−11	1926.9	1461.0	75.8	151.4	7.8	174.5	9.1	16.4	0.85	20.7	1.07	22.0	1.13
	RC		+ 3	1728.0	1330.6	77.0	130.1	7.5	162.0	9.4	13.2	0.76	23.1	1.35	2.7	0.15
8/15	3—P	2	− 1	1546.7	1164.3	75.3	134.1	8.6	118.2	7.6	13.6	0.88	13.1	0.84	16.6	1.07
	RC		+23	1182.7	939.4	79.4	62.7	5.3	112.5	9.5	9.0	0.76	14.7	1.25	0.9	0.07

8/17	3—P	2	+ 9	1782.6	1331.9	74.8	176.9	9.4	164.9	9.3	18.8	1.06	12.0	0.67	25.9	1.45
	RC		+21	1364.1	1067.8	78.3	77.0	5.6	138.5	10.1	7.4	0.54	15.3	1.12	3.3	0.24
8/20	3—P	3	+15	3000.6	2288.2	76.2	354.2	11.8	300.1	10.0	30.6	1.02	19.7	0.66	26.2	0.87
	RC		+23	2607.8	2058.0	78.8	159.8	6.1	226.2	8.6	16.8	0.64	24.5	0.94	5.8	0.22
8/22	3—P	2	+ 5	2018.1	1515.5	75.1	189.7	9.4	186.0	9.2	19.3	0.95	10.3	0.51	17.7	0.84
	RC		+11	1736.8	1363.2	78.5	93.4	5.4	138.2	8.0	11.4	0.65	15.6	0.89	6.6	0.38
8/24	3—P	2	− 2	1936.2	1488.9	76.9	181.5	9.4	133.2	6.9	18.4	0.95	9.8	0.51	14.0	0.72
	RC		+16	1344.8	1053.1	78.3	83.1	6.2	132.5	9.1	8.8	0.65	11.2	0.83	2.6	0.12
8/26	1—P§	1	−18	170.1	131.9	77.5	10.0	5.9	22.7	13.4	0.4	0.23	2.5	1.46	4.9	2.87
	RC		+ 3	215.2	168.7	78.4	6.4	2.9	35.2	16.3	0.7	0.32	2.5	1.16	0.8	0.3
8/27	2—P	3	− 5	2470.2	1862.2	75.4	265.5	10.7	240.8	9.7	22.5	0.91	15.8	0.64	16.0	0 65
	RC		+23	1558.0	1222.8	78.5	73.2	4.7	231.0	14.8	11.0	0.70	17.2	1.10	4.8	31
8/30	2—P	3	+11	2114.1	1603.0	75.8	237.4	11.2	194.5	9.2	7.5	0.35	6.7	0.31	6.5	0.31
	RC		+28	1827.5	1560.3	85.6	90.6	4.9	134.4	7.3	4.3	0.23	9.2	0.50	2.3	0.12
9/2	2—P	3	+ 6	1950.7	1619.6	83.0	206.2	10.5	113.0	5.8	16.5	0.84	11.1	0.56	10.9	0.57
	RC		+28	1887.7	1625.4	80.5	92.7	4.8	161.5	8.5	12.1	0.64	18.1	0.96	1.7	0.09
9/4	2—P	2	− 2	760.8	613.3	80.6	72.5	9.5	55.6	7.3	6.4	0.84	4.2	0.55	6.1	0.80
	RC		+ 5	486.1	390.4	80.3	19.7	4.1	53.9	11.1	3.9	0.80	5.0	1.03	2.5	0.50
9/6	2—P†	2	−15	1419.0	1060.6	74.8	146.6	10.3	169.1	11.9	14.8	1.04	8.0	0.56	12.4	0.87
	RC		+ 5	930.5	715.5	76.9	40.6	4.4	125.1	13.4	8.2	0.88	8.7	0.87	6.8	0.73

* A represents the animals which later received thyroxin and subsequently referred to as P.

* B represents the litter mates of A animals which were given the same ration and restricted to the same amount of food consumed by the A rats, and later referred to as RC (restricted controls).

§ Died during night of 8/26 leaving two groups in this series.

† Both P animals died on night of 9/7.

♯ During metabolism periods.

TABLE 3. INFLUENCE OF GLYCINE ON CREATINE-CREATININE EXCRETION IN HYPERTHYROIDISM

(Stock Diet No. 1)

Exp. No.	Animal marking and sex	Metabolism period	Food intake	Changes in body weight	Preformed creatinine	Creatine	Daily doses of *l*-thyroxin and glycine
		days	*gm.*	*gm.*	*mg.*	*mg.*	
4337	♀	8	73	−3	15.70	5.96	
	L♀	8	77	+7	12.37	4.53	
	♀	12	127	+13	32.42	42.23	0.3 mg. *l*-thyroxin.
	L♀	12	122	+15	36.72	52.55	0.3 mg. *l*-thyroxin and 100 mg. glycine.
	♀	10	150	+11	42.66	46.57	0.5 mg. *l*-thyroxin.
	L♀	10	152	+8	37.34	48.71	0.5 mg. *l*-thyroxin and 100 mg. glycine.
	♀	9	140	−14	42.89	65.20	1.0 mg. *l*-thyroxin.
	L♀	9	158	+5	35.47	61.34	1.0 mg. *l*-thyroxin and 100 mg. glycine.
Total*	♀	31	417	+10	117.97	154.02	
	L♀	31	432	+28	109.53	162.60	
	R♀	8	71	+4	17.73	7.66	
	D♀	8	72	+8	22.95	6.83	
	R♀	12	129	+4	39.60	49.41	0.3 mg. *l*-thyroxin.
	D♀	12	132	+10	42.55	55.81	0.3 mg. *l*-thyroxin and 100 mg. glycine.
	R♀	10	165	+20	25.93	41.81	0.5 mg. *l*-thyroxin.
	D♀	10	146	+7	36.37	63.57	0.5 mg. *l*-thyroxin and 100 mg. glycine.
	D♀	9	161	+1	33.69	55.39	1.0 mg. *l*-thyroxin.
	R♀	9	147	−1	34.92	70.37	1.0 mg. *l*-thyroxin and 100 mg. glycine.
Total*	R♀	31	455	+25	99.22	146.61	
	D♀	31	425	+16	113.84	189.75	
	DL♀	8	65	+4	15.74	4.53	
	DR♀	8	59	+5	16.20	5.90	
	DL♀	12	114	+12	30.88	32.77	0.3 mg. *l*-thyroxin.
	DR♀	12	131	+16	23.76	27.88	0.3 mg. *l*-thyroxin and 100 mg. glycine.
	DL♀	10	130	+5	27.34	31.58	0.5 mg. *l*-thyroxin.
	DR♀	10	147	+11	25.75	37.03	0.5 mg. *l*-thyroxin and 100 mg. glycine.
	DL♀	9	148	−2	34.46	52.48	1.0 mg. *l*-thyroxin.
	DR♀	9	154	+4	38.06	60.32	1.0 mg. *l*-thyroxin and 100 mg. glycine.
Total*	DL♀	31	392	+15	92.68	87.57	
	DR♀	31	432	+31	116.83	125.23	
Grand Total Thyroxinized animals		31	1264	+50	309.87	388.20	
Thyroxinized animals that received glycine		31	1289	+75	340.20	477.58	

* This represents the total experimental period during which thyroxin and glycine were administered.

ment. The allantoin was the only determination that gave us considerable trouble for weeks but all difficulties were overcome by a slight modification of the method of Harding and Young (17). The procedure we finally adopted is as follows.

Fifty cc. of the diluted urine was pipetted into a 500 cc. pyrex Kjeldahl flask and 7 cc. of concentrated HCl and 20 gm. of crystalline magnesium chloride was added. A reflux condenser, 60 cm. in length, preferably pyrex, was attached to the flask and with the condenser jacket empty, the mixture was allowed to boil gently for about 15 minutes until most of the liquid was evaporated. The hydrolysis was then interrupted and 3 cc. more concentrated HCl added. A Kjeldahl distilling head was placed on the top of the condenser to prevent loss of ammonia from hydrolysis of urea in the primary boiling, and boiling continued until frothing began. When very little frothing occurred, the concentration was continued until each drop of liquid falling back from the condenser caused a marked spattering of the contents of the flask. From this point a cold stream of water was allowed to circulate through the condenser and gentle boiling was continued for 2 hours. The second period of boiling hydrolyzed the allantoin. The total nitrogen of this hydrolysis mixture was determined by steam distillation. The allantoin nitrogen was calculated by difference from that obtained in this mixture and the combined ammonia and urea nitrogen.

Our departures from the original procedure of Harding and Young were as follows. We used 60 cm. instead of 45 cm. condensers, and 500 cc. Kjeldahl flasks instead of 200 cc. Erlenmeyer flasks. By using the larger size condenser and the long neck Kjeldahl flask, we dispensed with the large safety thistle tube and substituted a Kjeldahl distilling head. The significant modification we found essential is the addition of more hydrochloric acid after the primary boiling with the empty condenser jacket has proceeded to a point of reduction to small volume when an insufficient amount of acid has been left in the flask to prevent losses of ammonia. With such precautions, however, no difficulties need be experienced with the allantoin determination.

EXPERIMENTAL

The metabolism experiments of this study were carried out in two sereis. The rats were males taken from stock diet No. 1 (18). In Series I the animals were 65 days of age and weighed 148 to 156 gm. at the beginning of the investigation. In Series II the animals were 53 days of age and had initial weights of 93 to 97 gm. Each series consisted of 3 pairs of animals and in each pair one litter mate received subcutaneous injections of synthetic (*d-l*) thyroxin daily following a preliminary period of 12 to 14 days. The initial daily dose of thyroxin was 0.5 mg. which was increased after 5 days to 1 mg., the entire thyroxin metabolism period lasting about a month. The results submitted for blood and urine represent data obtained from 3 groups in each set until a thyroxinized animal died, thus leaving 2 groups for pooling of blood and urine. After 2 hyperthyroid animals died, results were available for individual groups. In every case the results secured on an individual group corroborated those obtained on pooled blood and urine from two or three groups.

Since in hyperthyroidism produced in the rat by thyroxin injection there is an increased nitrogen excretion in the urine, in order to make comparisons between controls and thyroxinized animals, it was necessary to calculate the excretions of all nitrogenous constituents in the urine as percentage of the total nitrogen; therefore, the uric acid, preformed creatinine, and creatine figures were recalculated and expressed as uric acid nitrogen, preformed creatinine nitrogen, and creatine nitrogen.

In order to eliminate the complication of exogenous metabolism, due to possible changes in protein intake, the controls were given the same amount of food daily, weighed to the accuracy of 0.1 gm. as that consumed the previous 24 hours by the animals injected with thyroxin.

Under our experimental conditions, depending on the diet and the amount of

food intake, we found the distribution of N.P.N. of the blood of albino rats of the Wistar Institute strain, to be as follows: total N.P.N., 35 to 90 mg.%; urea N, 20 to 30% of the total N.P.N.; uric acid, 2 to 3, occasionally as high as 4 mg.%; preformed creatinine, 0.5 to 2.5 mg.%; and creatine, 6 to 9, seldom over 9 mg.%. The amino acids gave too varying results to be of any significance and such determinations were, therefore, abandoned in the nitrogen distribution of the blood.

The only significant change in the non-protein nitrogen of the blood in hyperthyroidism of the rat we found was an increase in uric acid. This became apparent several days after the subcutaneous injections of thyroxin were begun. Such increase in uric acid concentration persisted until the termination of the experiments. In Series I there was an average increase of 45.1% and in Series II an average rise of 39.1% blood uric acid. To conserve space, such detailed data are deleted, particularly, since they are chiefly negative.

It is apparent from tables 1 and 2 that as the thyrotoxicosis advances there is, in addition to increased total nitrogen and creatine excretion, an increased ammonia and uric acid excretion. As the creatinura develops there is a decreased excretion of preformed creatinine. During the later stages of the hyperthyroidism there is an appreciable reduction in the excretion of allantoin. These changes have been calculated as percentage of the total nitrogen. That the results obtained from several groups represent each specific group studied is apparent from the data shown for the individual animals at the end of the tables. The results are consistent in both series of experiments. During the period of thyroxin administration, calculated as percentage of the total nitrogen, there was an increased excretion of 81.0% of ammonia nitrogen, an increased excretion of 39.2% uric acid nitrogen, a decreased output of 34.1% preformed creatinine nitrogen, a decreased excretion of 10.1% allantoin nitrogen, and an increased excretion of 303% creatine nitrogen. The most pronounced change, therefore, was in the creatine and the least change in the allantoin excretion. There were no noteworthy changes in the urea nitrogen excretion, since the amount of food ingested was the same in the pathological as in the control animals. Changes in the excretion of nitrogenous constituents of urine of the thyroxinized animals during the last week of the experiments, compared with the litter-mate controls, were as follows: ammonia N, +132.2%; uric acid N, +44.7%; preformed creatinine N, −42.9%; allantoin N, −15.8%; and creatine N, +125.0%. It is thus apparent that with the advance of hyperthyroidism there is a pronounced increased urinary excretion of ammonia, a small increase in uric acid, appreciably larger decrease in excretion of allantoin and a remarkable fall in excretion of creatine, when comparisons are made on the basis of percentage of total nitrogen excreted.

Influence of glycine on creatine-creatinine metabolism in hyperthyroidism. Since Beard and Pizzolato (19) have recently reported that the administration of glycine, peptids of glycine, as well as other amino acids, for a period of 1 to 2 days to the rat, are followed by increase in muscle creatine up to 138%, a study was made of the possible influence of glycine in creatine-creatinine metabolism in hyperthyroidism. A metabolism study was carried out on 6 rats on our stock diet No. 1 (18). The urinary output of preformed and total creatinine was taken daily with the exception of Sundays. The first 8 days served as a preliminary period. The animals were then arranged in 3 pairs. During the first metabolism period one animal was injected daily with 0.3 mg. *l*-thyroxin[2] and a litter mate with the same amount of *l*-thyroxin and 100 mg. glycine. During the second metabolism period of 10 days, the same procedure was followed but the daily dose of thyroxin was increased to 0.5 mg. And during

[2] Kindly furnished by Dr. John F. Anderson of Squibb's Institute for Medical Research.

the third metabolism period a similar technic was adopted but the daily thyroxin dose was increased to 1.0 mg. At the termination of the experiments creatine determinations were made of the muscle and heart on the dry as well as the moist basis. It will be noted from table 3 that the injection of 100 mg. glycine daily to thyroxinized rats produced a small increase in urinary excretion of preformed creatinine and a significant increase of creatine. The average for the entire metabolism period was an increase of 9.7% in excretion of preformed creatinine and an increase of 23.2% in excretion of creatine. Glycine, however, exerted no protective influence on the creatine content of the muscles and the myocardium. There was a reduction of 20 to 30% in muscle creatine and 40 to 50% in heart creatine in the animals that received glycine and thyroxin as well as those that received thyroxin alone. Our results are in harmony with those found by Bodansky and Duff (20).

SUMMARY

Subcutaneous daily injections of synthetic (*d-l*) thyroxin produce a disturbance in nitrogenous metabolism, as evidenced not only by an increased total nitrogen output in the urine and a marked creatinuria and reduction of preformed creatinine but also by pronounced increases in ammonia and uric acid excretion and by an appreciable reduction in the excretion of allantoin. Thyroxinized animals show a marked retention of blood uric acid. The marked reduction of muscle and heart creatine in hyperthyroidism cannot be prevented by massive doses of vitamin A or ascorbic acid.

Subcutaneous injections of glycine are followed by small increases in urinary excretion of preformed creatinine and in significant increases in excretion of creatine, but glycine will not prevent the great losses of creatine from the muscles and the myocardium.

REFERENCES

1. Hunter, A.: Creatine and Creatinine, 1928, Longmans, Green & Company; Pugsley, L., E. Anderson and J. B. Collip: *Biochem. J.* 28: 1135. 1934.
2. Bodansky, M., and V. B. Duff: *Endocrinology* 20: 822. 1936.
3. Allison, J. B., C. Glaser and S. L. Leonard: *Proc. Soc. Exper. Biol. & Med.* 42: 491. 1939.
4. Drill, V. A.: *J. Nutr.* 14: 355. 1937.
5. Shorr, E., H. B. Richardson and H. G. Wolff: *J. Clin. Invest.* 12: 966. 1933.
6. Bodansky, M.: *J. Biol. Chem.* 109: 615. 1935.
7. Berg, H.: *Arch. f. d. ges. Physiol.* 185: 359. 1937.
8. Fischer, G. G., and C. Oehme: *Klin. Wchnschr.* 16: 1453. 1937.
9. Sure, B., and R. M. Theis: Unpublished data.
10. David, C.: *Ztschr. f. d. Ges. Exp. Mediz.* 104: 397. 1938.
11. Borsook, H.: *J. Biol. Chem.* 110: 421. 1935.
12. Borsook, H., and J. W. Dubnoff: *J. Biol. Chem.* 131: 163. 1939.
13. Evelyn, K. A.: *J. Biol. Chem.* 115: 63. 1936; 117: 365. 1937.
14. Sure, B., and V. M. Wilder: *J. Lab. & Clin. Med.* 26: 874. 1941.
15. Van Slyke, D. D., and G. E. Cullen: *J. Biol. Chem.* 24: 117. 1916.
16. Folin, O.: *J. Biol. Chem.* 17: 469. 1914.
17. Harding, V. J., and E. G. Young: *J. Biol. Chem.* 40: 227. 1919.
18. Sure, B.: *J. Biol. Chem.* 69: 65. 1926.
19. Beard, H. H., and P. Pizzolato: *J. Biochem.* 28: 421. 1938.
20. Bodansky, M., and V. B. Duff: *Proc. Soc. Exper. Biol. & Med.* 34: 307. 1936.

INFLUENCE OF IODINE FEEDING ON THE NORMAL THYROID WITH REFERENCE TO ITS RELATIVE POTENCY IN STIMULATING METABOLISM AND HEART RATE

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IN CONTINUATION OF OUR RESEARCH on the relationship between the two effects produced by thyroid material, namely increase of metabolism and acceleration of heart rate, when tested in thyroidectomized rats, we were able to show that the variability of the influence on the heart rate observed with various thyroid preparations (1) is apparently not produced by differences in the handling of the glandular material postmortem, to wit by autolysis or bacterial decomposition, but is caused by an original difference in the glands themselves. While fresh pig's thyroid as well as normal human thyroid did not accelerate the heart beat of the thyroidectomized rat significantly at a dosage producing a notable metabolic increase, glands obtained by surgery from thyrotoxic patients affected the heart rate to a high degree even when the quantity given to the test rat was kept so small that the metabolic response remained at a low level (2).

In contrast to these observations a gland from a hyperthyroid patient, who had not been subjected to iodine medication preceding surgical intervention, was lacking in this action on the heart. Because of the common pre-operative practice of giving thyrotoxic patients iodine it is difficult to obtain non-iodized toxic goiter glands and for this reason we have as yet been unable to duplicate our last experience and thus confirm the findings.

It was our hypothesis, advanced tentatively, that the heart accelerating substance, in consonance with the metabolic hormone, is produced but not accumulated in the hyperactive gland and rather it is immediately released into circulation. Iodine medication would cause an accumulation of both factors in the thyroid, thus reducing the amount available to the organism and consequently causing a decrease in metabolic as well as heart rate.

It would seem, however, that the normal gland does not keep the cardiotropic factor stored in the same proportion as the metabolic hormone, leaving the question open whether it is a product of normal secretion of the gland, i.e. a second hormone, or the result of pathologically changed activity and therefore found only in diseased thyroids.

If iodization causes a storage of the cardiotropic substance in the pathologic gland similar results might be expected in the normal gland. This assumption can readily be

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checked experimentally and such is the object of the work presented in this publication.

The literature presents a number of studies on the influence of iodine on the normal thyroid, dealing with various aspects of the histology, chemistry and physiology of the gland.

Marine and Lenhart (3) concluded from histological changes that potassium iodide feeding reduces thyroid function in the dog. Since the same histological picture, however, may be observed in opposite functional states of the thyroid, these results contribute little suggestive material to our problem.

Gutman, Benedict, Baxter and Palmer (4) have found that iodine medication increases the iodine content of the gland as well as its absolute and relative thyroxine content. This obviously augments the quantity of metabolism stimulating substance contained in the gland, possibly as a result of increased production, or of accumulation by decreased release, or both. In the second case, that of decreased liberation of the hormone by the gland, there ought to be a drop in metabolic rate following iodine treatment, provided this sparing action is not overshadowed by increased production.

The work done with respect to change in metabolism brought about by iodine treatment did not lead to uniform results. Hildebrand's (5) investigations on 3 normal and 2 thyroidectomized rats cannot be considered as convincing, even if the findings had been more consistent than they actually were. Neither do the varying observations on 2 dogs, presented by Salvadori (6), contribute any closer information to this question. Liebert and Linton (7) observed an occasional metabolic increase after iodine medication. From the work of Marine, Deutsch and Cipra (8) it appears that increase, decrease, or no change may follow iodine medication, with no definitely suggestive relationship to the condition of the thyroid preceding treatment.

It is evident that the literature does not offer any information that would lend support to our expectation to find both factors similarly affected by iodine treatment in the normal and hyperthyroid gland.

METHOD

As objects of assay we used thyroidectomized rats, following our method as described previously (9).

The glandular material used in the first part of our investigation was obtained from rats by thyroidectomy. The control donors were untreated male albino rats which were thyroidectomized at an age of 76 to 84 days. The right and left lobes were collected separately and weighed. The lobes from one side were used for iodine determination, the other lobes were fed to the assay animal on 3 consecutive days. For equal treatment 3, or multiples of 3, thyroid lobes, up to 12, were used at a time. The treated donor rats received 5 mg. of iodine in form of Lugol's solution 6 times weekly, beginning at an age of 60 days and carried through to the day of thyroidectomy, performed within the same age limits as given above. In view of the higher iodine content of these glands only 3 lobes, one per day, were used.

For the determination of iodine, none of the usual micro-methods, as far as tried by us, was found satisfactory. Being developed for analysis of blood and tissues of low iodine content, they are adapted to the destruction of large quantities of organic matter, but the accuracy, usually sufficient for the purpose, did not measure up to our needs, at least not in our hands. We found that Kendall's method could well be adapted to microchemical procedure by reducing the quantities of alkali in the melting process, and of the other reagents in proportion to the small amount of organic matter. The melting process was performed in a small Pyrex test tube, avoiding carefully all overheating. If this precaution is observed, the glass is practically not attacked, at least not to an extent to interfere with the analysis. In every instance a blank was run, which was always slightly positive in spite of the use of the purest reagents available.

No claim is made that right and left lobes were absolutely equal. Moderate dif-

ferences in weight were usually found, which may correspond to differences in size of the lobes, but the variations in the division of the isthmus and the occasional adherence of particles of non-glandular material probably were influential also. In view of this, the iodine determinations obtained from the opposing lobes can serve only as an approximate guide to estimate the doses of thyroid iodine applied.

With the object of obtaining an estimation as to what extent the two lobes correspond to each other, 2 groups of rats were investigated with the following results:

1. Four rats 80 to 82 days old: weight 186 to 210 gm.; right lobes 24.0 mg. weight, containing 3.14γ iodine; left lobes 24.0 mg., containing 2.69γ iodine.
2. Five rats 77 to 82 days old: weight 173 to 193 gm.; right lobes 38.5 mg. with 4.49γ iodine; left lobes 36.0 mg. with 4.72γ iodine.

It may be mentioned that the difference in the weight of the lobes in the actual tests was mostly somewhat more significant than in these two examples.

Analogous tests, as with rats, were performed with rabbits' thyroids. The iodized rabbits received 15 mg. iodine daily for about 3 weeks preceding thyroidectomy; all were males and between 4 and 5 months old at the time of operation. In view of the larger size of the gland, the lobe used for treatment was cut into parts as equal in size as was feasible, to give proportionate quantities of material at each feeding. The determination of metabolic rate and heart rate following treatment was performed on the fifth day. The results obtained are compiled in table 1.

As in our previous work on fresh glands, we do not lay any stress on proportionality between iodine content and metabolic response. The iodine determination of right lobes, performed on the whole group summarily, does not give an accurate measure with regard to the daily administration of the corresponding left lobes; in the rabbit gland, an equal division of the material into minute parts of a few milligrams of weight necessarily yields only a rough approximation. The relation between metabolic increase and change in heart rate in the test animal is the essential point of interest.

It can easily be seen that the thyroids from all untreated animals, rats as well as rabbits, have a relatively weak stimulating effect on the heart, whereas this potency is considerably increased in the iodized animals. In the case of the first iodized rabbit, the test rats 377 and 379 received their treatment with the thyroid lobe before the iodine determination in the other lobe had been finished. Since an iodine content as high as 142γ had not been anticipated, the lobe had been divided between only two rats, leading to a considerable overdosage. In view of this fact, the exceptionally high heart rates obtained might not be considered as fully convincing. However, in our previous work (2) we gave doses of still higher iodine content of non-heart-stimulating normal human thyroid, and obtained correspondingly high metabolic responses concomitant with low action on the heart (maximum 6γ iodine per 10 gm. weight per day giving 59% metabolic increase, but a change of heart rate from 193 to 222).

DISCUSSION

The experiments quite clearly demonstrate that iodine causes an accumulation in the thyroid of the substance that stimulates the heart rate. Whether this substance is produced at a higher rate or only kept away from circulation and thus stored, cannot be decided upon. Although the increase appeared secondary to the increase of the iodine in the gland and to the increase in metabolic potency dependent on it, the heart stimulating activity increases at a much faster rate. Incidentally, it was observed that in practically every instance the iodized thyroids were smaller in size than the normal glands if compared in relation to body weight of the animal.

Our attempts to duplicate on guinea pigs and rabbits as test objects the results obtained on rats have remained without success. The determination of basic heart rate, i.e. the count at perfect rest, is extremely difficult in these animals as they are too excitable. Also the drop after thyroidectomy is not as conspicuous and uniform

TABLE I

No. of test rat	Days of iodization	Total of half-thyroids given	Total γI	γI per 10 gm. wt. per day	Metabolism mg. O_2 per kg. per hr.			Heart rate per min.	
					Before	After	% incr.	Before	After
Thyroids from Normal Rats									
59		3	5.35	0.105	960	1065	11	196	214
138		12	28.1	0.5	900	1280	42	198	205
143		12	14.7	0.29	940	1195	27	195	215
198		9	8.5	0.20	900	1160	29	195	218
241		12	20.0	0.37	1085	1235	14	197	194
Thyroids from Iodized Rats									
63	23[2]	3	20.7	0.4	920	1090	19	196	290
109	23[2]	3	24.0	0.5	900	1105	23	195	260
195[1]	17[2]	3	12.6	0.21	1100	1135	3	198	225
199	23[2]	3	10.6	0.175	1000	1050	5	195	320
240	20[2]	3	17.4	0.41	1150	1470	28	195	297
235	23[2]	3	15.0	0.33	960	1175	22	192	240
292	26[2]	3	20.0	0.44	1100	1390	26	198	278
344	26[2]	3	18.0	0.37	1140	1390	22	193	272
347	22[2]	3	17.0	0.42	1080	1390	29	196	310
365	22[2]	3	22.0	0.46	930	1295	39	196	310
406	20[2]	3	17.0	0.33	905	1105	22	198	240
382	20[2]	3	15.0	0.25	1000	1090	9	200	275
Rabbit Thyroids									
376	None[3]	Total	50	0.8	1010	1250	24	192	216
367	None[3]	Total	36	0.7	995	1425	43	195	215
459	None[3]	Total	26.7	0.52	900	1210	34	197	210
377	19[2]	½	71	1.3	900	1425	58	193	350 over
379	19[2]	½	71	1.75	1010	1600	59	196	400
366	21[2]	⅛	30	0.57	925	1195	29	192	261
374	21[2]	⅛	30	0.67	940	1195	27	194	300
381	21[2]	⅛	30	0.77	1100	1400	27	196	280
404	21[2]	⅛	30	0.56	900	1040	16	197	255
383	21[2]	⅛	30	0.42	1100	1275	16	193	280

[1] This animal refused to eat the thyroid medication. The ration was placed far back into its mouth to insure ingestion, but it is not impossible that perhaps once or twice the gland was spit out later on. That would explain the low response in metabolism as well as heart beat.

[2] Donors.

[3] Rabbit.

as in the rat. The values obtained in normal and thyroidectomized guinea pigs were frequently overlapping.

From clinical observations obtained on patients treated with thyroid globulin, of low cardiotropic action in the rat, the impression is gained that thyroid globulin exerts less effect on the nervous system than whole thyroid powder. This suggests the possibility that the heart acceleration may be the result of a sympathetic stimulation, which would be in agreement with Oswald's (10) statement that thyroxine differs from whole thyroid in that it lacks the sensitizing effect on the sympathetic system. The involvement of the adrenals in Graves' disease, as claimed by Marine (11), might find its explanation on that basis. Unfortunately, Oswald's investigation is far from

proving his claims. His conclusions are based on the alterations observed in the pressor response of the anesthetized animal to adrenalin or vagal stimulation by a preceding intravenous injection of dissolved thyroid globulin. Since an identical, albeit weaker effect was obtained with other proteins, his results can be interpreted as characteristic for proteins and varying in intensity with the type of same, but not as specific for thyroid. It must be emphasized also that the effect of thyroid is long lasting but slow to set in and consequently his experimental set-up investigating "acute" effect was not adequate for the intended purpose. It is evident that we cannot take advantage of his experiments to arrive at a theory on the mechanism of the heart stimulation observed in our research.

SUMMARY

The thyroids of normal rats and rabbits, when fed to thyroidectomized rats at dosages causing conspicuous metabolic increment, do not affect the bradycardia significantly. If the rats or rabbits receive a daily treatment with 5 or 15 mg. of iodine respectively for two to three weeks, the iodine content of their thyroids increases considerably. Doses of these glands, having an iodine content in the same range as employed with untreated thyroid, caused in the thyroidectomized rats metabolic stimulation within the same limits. The effect on the heart rate was increased at an unproportionately higher degree, so that fast heart rates were obtained at moderate metabolic levels.

It is concluded that iodine feeding causes an accumulation in the thyroid of a substance that accelerates the heart beat.

REFERENCES

1. Meyer, A. E., and M. Yost: *Endocrinology* 24: 806. 1939.
2. Meyer, A. E., and M. Thompson: *Endocrinology*, 27: 917. 1940.
3. Marine, D., and C. H. Lenhart: *Arch. Int. Med.* 4: 440. 1909.
4. Gutman, A. B., E. M. Benedict, B. Baxter and W. W. Palmer: *J. Biol. Chem.* 97: 303. 1932.
5. Hildebrand, F.: *Arch. Exper. Path. & Pharmacol.* 96: 292. 1923.
6. Salvadori, C.: *Biochem. J.* 245: 314. 1932.
7. Liebert, W. J., and C. S. Linton: *J. Pharmacol. & Exper. Therap.* 53: 418. 1935.
8. Marine, D., M. Deutsch and A. Cipra: *Proc. Soc. Exper. Biol. & Med.* 24: 675. 1927.
9. Meyer, A. E., and A. Wertz: *Endocrinology* 24: 683. 1939.
10. Oswald, A.: *Rev. Franç. d'Endocrinol.* 5: 254. 1927.
11. Marine, D.: *Am. J. Med. Sc.* 180: 767. 1930.

THE BLOOD PICTURE OF ADRENALECTOMIZED ANIMALS TREATED WITH DIFFERENT ADRENAL FRACTIONS[1]

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THE LITERATURE on the blood picture of adrenalectomized animals receiving adequate substitutional therapy is very meager. As pointed out by Waterman et al. (1) (1939) in their review of the literature the earlier studies were made before potent extracts were available, hence the changes were complicated by post-operative reactions. An increase in hemoglobin and the number of red blood cells was observed by the majority of the early workers.

Zwemer and Lyons (2) found a relative neutropenia and lymphocytosis and Corey and Britton (3) observed a relative leucocytosis with the development of adrenal insufficiency. Harrop (4) reported that in one dog the mean corpuscular volume increased from 61.3 to 75.2 cu. μ. during insufficiency. He attributed this to a shift of body water. Waterman et al. (1) found "no important changes in either the red or white blood cell picture before and after adrenalectomy" when the dogs were maintained on 'cortin.' They observed an increase in 'hematocrit' in only 2 out of 5 cases of severe insufficiency.

The present studies were carried out in order to determine the effect of various adrenal preparations on the blood picture of the adrenalectomized cat and dog. The plasma electrolytes of two of the dogs (1*M* and 2*M*) studied, were reported in an earlier paper (5) and may be referred to as an index of the clinical condition of the animals.

METHODS

The preparation of the various adrenal extracts used has been described in an earlier paper (5). In the study of their effect on the adrenalectomized cat and dog complete blood counts have been made. Studies were made before operations so that each animal served as his own control. All of the animals were adults in good health at the time the investigations were started. The animals had been in the laboratory several weeks and were thoroughly adjusted to their surroundings.

The dogs were maintained on a constant diet of beef heart and Purina Dog Chow while the cats were fed a constant diet of beef heart and salmon. All animals received haliver oil capsules weekly. Cats received vitavose, in addition.

Blood was drawn from the jugular vein of the cat and the radial vein of the dog, care being taken to avoid stasis. All samples were taken in the morning before the animals were fed. Heparin was used as anticoagulant.

Hemoglobin was determined in a Haden-Hausser hemoglobinometer, the stand-

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ardization of which was checked by the Haldane method. The blood for the red blood cell counts was diluted with Hayem's solution in Thoma pipettes which were approved by the U. S. Bureau of Standards. The cells were counted in 5 squares (each square representing one twenty-fifth of a sq. mm. in surface area) of the Levy-Hausser counting chamber (improved Neubauer ruling). The blood for the white blood cell counts was diluted with tenth normal hydrochloric acid (6) in Thoma pipettes. The number of cells in 4 squares (each square representing 1 sq. mm. of surface area) of the Levy-Hausser counting chamber was determined. Differential counts

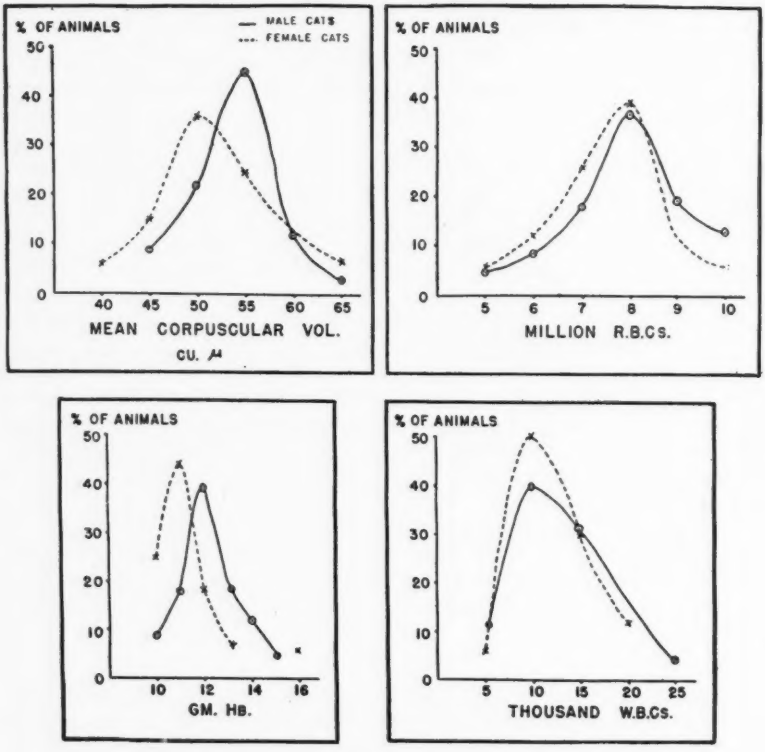

Fig. 1. The blood count of normal male and female cats.

were made of 100 cells stained with Wright's stain; however, if the leucocyte count was above 12,000 per cu. mm., 200 cells were counted. The volume per cent of packed red blood cells was determined by the hematocrit method of Wintrobe (7).

Blood volume determinations were made on the dogs. The method of Gibson and Evans (8) using the blue dye T-1824 was employed.

Bone marrow of the cats was studied when the 'insufficiency' determinations were made. Bone marrow from other normal cats in this laboratory was used for control.

RESULTS

Twenty-three normal male cats and 16 normal female cats were studied. Figure 1 shows the distribution curves for the red blood cell counts, the hemoglobin value,

TABLE I. LEUCOCYTE AND DIFFERENTIAL BLOOD CELL COUNTS OF NORMAL CATS

	Leucocytes	Neutrophils			Eosinophils	Lympho-cytes	Mono-cytes
		Juveniles	Band	Segmented			
	1000/cu. mm.	%	%	%	%	%	%
Females							
Average	12.9	0.2	13.3	34.4	4.4	45.4	2.3
Range	5.85–20.2	0–2.0	4–22	20–50	2–12	22–62	0–7
Males							
Average	11.26	0.1	6.9	39.3	5.2	46.7	1.8
Range	6.2 –24.5	0–2.0	0–26	6–68	0–15	22–76	0–7

the mean corpuscular volume and the white blood cell levels for the two sexes. Table 1 gives the differential blood cell counts for the two sexes. The hemoglobin value, red blood cell count and volume of packed red blood cells was definitely higher in the male than in the female cats averaging 12.3 gm. per 100 cc., 8.016 million per cu. mm. and 42.8% respectively as compared with 11.5 gm., 7.62 million and 40.4% in the females.

The average values for the 39 cats studied were: hemoglobin 11.98 gm. per 100 cc., red blood cells 7.85 million per cu. mm., white blood cells 11.93 thousand per cu. mm., volume of packed red blood cells 41.7% mean corpuscular volume 53.1 cu. μ.

Figure 2 shows the results obtained on *cat HS* before adrenalectomy and after operation when treated with different preparations. He appeared to be in excellent clinical condition except during adrenal insufficiency. The red blood cell count was

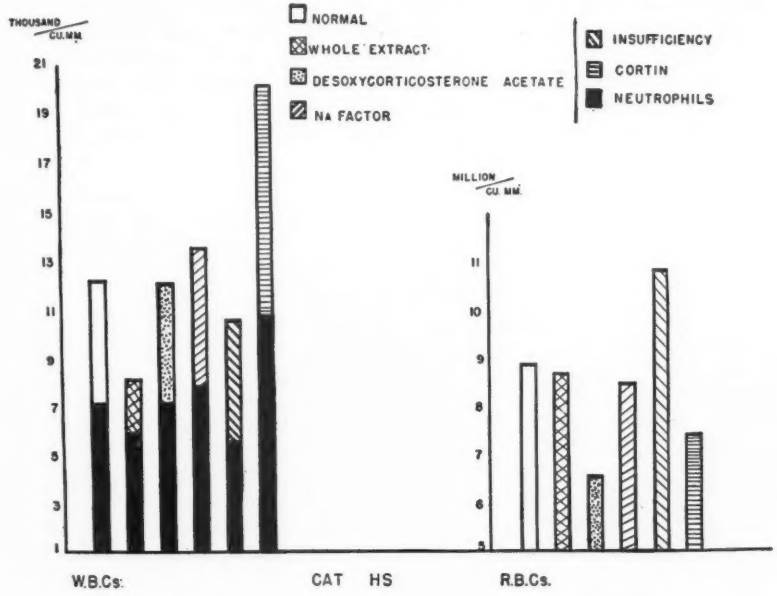

Fig. 2. THE BLOOD COUNT OF AN ADRENALECTOMIZED CAT WHEN TREATED WITH DIFFERENT ADRENAL PREPARATIONS.

TABLE 2. CHANGES IN RED BLOOD CELL COUNTS OF CATS DURING MILD ADRENAL INSUFFICIENCY

	Hb	R.B.C.	Packed red blood cells	Color index	Mean corp. vol.	Mean corp. Hb.	Mean corp. Hb. concn.
	gm./100 cc.	million/cu. cc.	vol. %		cu./μ	γγ	%
Normal values (Av. of 6 cats)	11.6	7.24	40.2	1.00	55.5	16.0	28.8
Mild Insufficiency Values (Av. of above 6 cats when insufficient)	13.0	8.66	46.0	0.93	53.1	15.0	28.2

normal while under treatment with whole extract or sodium factor. The somewhat lower value in the case of *HS* when on cortin may be due to the fact that at that time he appeared to have a worm infestation as indicated by a 20% eosinophilia. The very low count with desoxycorticosterone acetate can be accounted for by dilution from increased water retention. The increase in body weight indicated such retention. The high red blood cell count during insufficiency was undoubtedly due to a decrease in plasma volume. At no time did the white blood cell count show a significant change except during cortin treatment. This was true in 3 cats being 17,300, 18,850 and 20,200 respectively. A high percentage of eosinophils 8, 12, and 20% respectively was observed. (As noted above this was possibly due to a worm infestation.) In the other 2 cats the white blood cell count was normal (9,650 and 12,600) during cortin treatment and the differential blood cell count was within the normal range.

These results are typical of those obtained on the 5 cats thus treated. The averages were: a decrease from 41 to 39.5% in the volume of packed red blood cells with cortin treatment, an increase to 43.3% during insufficiency, and a decrease to 38% with desoxycorticosterone acetate treatment. There was no significant deviation from normal with sodium factor or whole extract treatment. The average white blood cell counts were: normal 11,900, whole extract treatment 11,950, sodium factor treatment 12,800, desoxycorticosterone acetate treatment 11,200, cortin treatment 15,900 and insufficiency 13,500 per cu. mm.

TABLE 3. CHANGES IN THE BLOOD COUNT DURING MILD ADRENAL INSUFFICIENCY

		Differential						
		Neutrophils						
	W.B.C.	Juvenile	Band	Filament	Eosin.	Lymph.	Mono.	Bone marrow
	1000/cc.	%	%	%	%	%	%	
Normal values								
Average of 6 cats	9.58		8.5	42	5.1	43.7	0.7	
Range	7.1–12.1		4–14	26–50	2–10	30–58	0–2	
Mild insufficiency values								
Average of above 6 cats when insufficient	9.57	0.2	11	36	7.5	42.3	3	Bone marrow of 5 cats was studied. In all cases slight hypoplasia was noted
Range	7.05–10.3	0–1	3–16	24–46	0–19	21–64	0–5	

Five male cats were studied when showing mild symptoms of adrenal insufficiency. Tables 2 and 3 give the normal and 'insufficiency' values. No significant variation occurred in either the leucocyte level or the differential blood cell count, but, an increase was noted in the red blood cell count, hemoglobin value and volume of packed red blood cells. The bone marrow was, in each of the 5 cases studied, slightly hypoplastic.

Six cats were studied when showing symptoms of terminal adrenal insufficiency. Table 4 summarizes the 'normal' and 'insufficiency' values of these animals. Four of the cats showed a definite decrease in the percentage of neutrophils, one showed no change and the sixth one showed an increase during insufficiency. In 3 cases the bone marrow was aplastic, in one it was markedly hypoplastic, in one slightly hypoplastic, while in the sixth case the bone marrow was within the normal range, but there was slight hyperplasia with tendency for regeneration.

TABLE 4. CHANGES IN THE BLOOD COUNT DURING TERMINAL ADRENAL INSUFFICIENCY

Cat	Hb		R.B.C.		W.B.C.		Neutrophils		Bone marrow Terminal insufficiency
	Norm.	Insuf.	Norm.	Insuf.	Norm.	Insuf.	Norm.	Insuf.	
	gm./100 cc.		*million/cu. mm.*		*1000/cu. mm.*		%	%	
HP	12.0	14.0	8.40	9.40	9.95	6.10	40	18	Marked hypoplasia
GT	11.5	13.0	8.16	8.90	11.20		38	67	General appearance of aplastic myeloid tissue
IW	11.0	13.0	6.84	8.75	9.50	9.20	38	29	Aplastic
IT	14.0	16.0	10.50	11.55	6.20	17.65	50	54	Aplastic
JE	12.0	16.5	7.32	11.50	8.50	14.10	46	28	Within the normal range, but a slight hyperplasia with tendency for regeneration
DQ	11.0	12.0	7.30	8.14	14.30	4.40	55	18	Slight hypoplasia

Two male dogs and two female dogs were studied before adrenalectomy and after operation when treated with whole adrenal extract, cortin, sodium factor and desoxycorticosterone acetate. Two of them were also studied when maintained on sodium salts without any hormone therapy. Blood volume determinations were made on 3 of the dogs.

Figure 3 shows the results on *dog 2M* and is typical of the findings on the 4 dogs studied. When the dog is well maintained after adrenalectomy, whether on whole adrenal extract, cortin, or sodium factor, there is little or no variation from the normal blood picture. These observations are in agreement with those of Waterman et al. (1) on dogs treated with whole adrenal extract. When treated with desoxycorticosterone acetate there was evidence of hemodilution as judged by the red blood cell count and blood volume values. The average of 15 leucocyte counts when *dog 2M* was on whole adrenal extract was 15,050 per cu. mm. This is somewhat higher than the preoperative control values and the postoperative values when he was receiving other treatment. This may have been due to the fact that the whole extract was not as highly purified as the other preparations used.

When *dog 1M* was carried on sodium salts his red blood cell count was 8.43 million per cu. mm., white blood cell count 11.65 thousand per cu. mm. and neutrophils 40%. The preoperative control values were 9.14 million, 9.20 thousand and 44% respectively. The blood picture of the adrenalectomized animal carried on sodium salts alone is essentially unchanged from the normal.

DISCUSSION

The normal red blood cell counts of the cats observed in this laboratory compare closely with those reported by Ackart et al. (9). The average figures reported by them were: red blood cell count 7.24 million per cu. mm., hemoglobin, 11.22 gm. per 100 cc., volume of packed red blood cells 40.15%.

The average white blood cell count reported by Ackart et al. (9) was, however, somewhat higher being 15,000 per cu. mm. Likewise they observed a higher percentage of neutrophils, 59.0%.

Modifications in the blood picture of the adrenalectomized cat or dog during in-

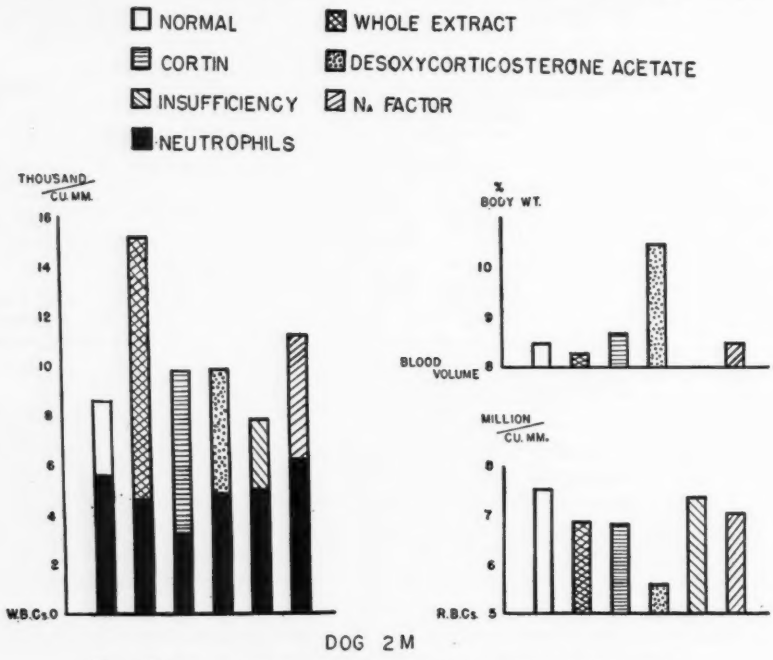

Fig. 3. The blood volume and blood count of an adrenalectomized dog treated with different adrenal preparations.

sufficiency appear to be due primarily to a decrease in plasma volume. The increase in red blood cell count and hemoglobin value in the insufficient cat was not accompanied by any significant change in the color index or mean corpuscular hemoglobin concentration. In the case of the dogs the importance of the change in plasma volume in modifying the red blood cell count and hemoglobin value is clearly shown by the blood volume decrease.

The increase in plasma volume during desoxycorticosterone acetate treatment was probably due to over-dosage. Ferrebee et al. (10) reported edema in some cases when an over-dosage of desoxycorticosterone acetate was used. As judged by the plasma electrolyte levels, the amount of acetate in excess of the requirement for complete maintenance was small. The dogs showed none of the symptoms of excessive over-dosage noted by Ferrebee.

It is interesting to note that with the development of insufficiency the hypoplasia of the bone marrow progressively increased. The cats which were studied when showing only mild insufficiency showed slight hypoplasia of the bone marrow, while terminal insufficiency was usually accompanied by marked hypoplasia. It is probable that the changes observed are merely a further index of the general functional state of the animal and are occasioned by the progressive decrease of normal physiological activity.

Prolonged starvation is accompanied by the development of a hypoplastic condition of the bone marrow. Five normal cats had all food withheld for 4 weeks. At the end of this period the bone marrow of one was aplastic, of two markedly hypoplastic and of the remaining two slightly hypoplastic. It is unlikely, however, that starvation was of importance in causing the bone marrow changes of the cats showing adrenal insufficiency. While some of them showed a loss of appetite for some time, in the majority of cases food was completely refused for two days or less.

SUMMARY

The normal red blood cell count, white blood cell count, hemoglobin value and differential blood cell count and volume of packed red blood cells of 23 normal male cats and 16 normal female cats were determined. The red blood cell count and hemoglobin value and volume of packed red blood cells of the males were significantly higher than those of the females.

Adrenalectomized cats and dogs when well maintained showed an essentially normal blood picture regardless of the type of treatment they were receiving. In some cases with desoxycorticosterone acetate therapy there was definite evidence of hemodilution. In the adrenalectomized cat showing mild symptoms of adrenal insufficiency slight hypoplasia of the bone marrow and hemoconcentration were noted. In the terminal stages of adrenal insufficiency marked hypoplasia of the bone marrow was usually found. In several cases a marked decrease in the percentage of neutrophils was also observed.

I wish to thank Doctor F. A. Hartman for the operations he performed on my animals and Doctor R. A. Knouff for the bone marrow studies. I am also indebted to Doctor R. D. Shaner of Hoffman-La Roche for the desoxycorticosterone acetate used in these experiments.

REFERENCES

1. Waterman, L., I. E. Uyldert, J. Thomassen and F. Oestreicher: *Endocrinology* 25: 885. 1939.
2. Zwemer, R. L., and C. Lyons: *Am. J. Physiol.* 86: 545. 1928.
3. Corey, E. L., and S. W. Britton: *Am. J. Physiol.* 102: 699. 1932.
4. Harrop, G. A.: *Bull. Johns Hopkins Hosp.* 59: 11. 1936.
5. Hartman, F. A., L. A. Lewis, J. E. Gabriel, H. J. Spoor and K. A. Brownell: *Endocrinology* 27: 287. 1940.
6. Boerner, F.: *Bull. Am. Soc. Clin. Lab. Tech.* 1: 55. 1935.
7. Wintrobe, M. M.: *Am. J. Med. Sc.* 185. 58. 1933.
8. Gibson, J. G., and W. A. Evans: *J. Clin. Invest.* 16: 301. 1937.
9. Ackart, R. J., J. S. Shaw, Jr. and J. S. Lawrence: *Anat. Rec.* 76: 357. 1940.
10. Ferrebee, J. W., C. Ragan, D. W. Atchley and R. F. Loeb: *J.A.M.A.* 113: 1725. 1939.

VOLUNTARY INTAKE OF CALCIUM AND PHOSPHORUS IN PARTIALLY NEPHRECTOMIZED AND PARATHYROIDECTOMIZED RATS

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THE RECENT EXPERIMENTS of Richter and Eckert (1, 2) indicate that the metabolism of calcium and phosphorus may be regulated in part by voluntary selection of these materials from the diet. In parathyroidectomized rats an increased amount of calcium and a decreased amount of phosphorus was consumed. This compensatory mechanism was sufficient to prevent or arrest manifestations of tetany. Further, this altered voluntary intake could be reversed by subsequent transplantation of parathyroid tissue.

From these results it would seem reasonable to expect that in other conditions in which calcium and phosphorus metabolism is disturbed, alterations in their voluntary intake might be demonstrable. One such condition is chronic renal insufficiency. The evidence that such a disturbance may occur in this condition is as follows.

(*a*) In renal insufficiency phosphorus excretion through the kidney is decreased, while its excretion in the intestine in the form of calcium phosphate is increased (3).

(*b*) In the advanced or terminal stages of chronic nephritis hyperphosphatemia (4) and hypocalcemia (5) are frequently demonstrable.

(*c*) Metastatic calcification of soft tissues such as lung parenchyma is sometimes observed in human cases of advanced nephritis with renal insufficiency. In partially nephrectomized rats Donohue, Spingarn and Pappenheimer (6) observed an increased calcium content of the remaining renal tissue.

(*d*) In a number of cases of chronic nephritis marked demineralization of the skeleton and lesions simulating osteitis fibrosa cystica have been described (7–10).

(*e*) It has been repeatedly observed that in protracted renal insufficiency the parathyroid glands become enlarged and hyperplastic. These observations have been confirmed experimentally in rats by Pappenheimer (11, 12) and his associates. The literature on this subject has recently been reviewed (13).

The evidence we wish to present that the voluntary consumption of calcium and phosphorus may be altered in renal insufficiency is entirely circumstantial. It has been established that the degree of parathyroid hyperplasia in renal insufficiency is roughly proportional to the severity of the renal impairment in man (14) and in the rat (12). Yet marked demineralization in adult chronic nephritis is rarely encountered and in those exceptional cases in which it has been described there is no suggestion that the degree of skeletal involvement was dependent on the severity of renal insufficiency. Although Ginzler and Jaffe (15) have described more constant osseous changes in renal insufficiency, these were osteosclerotic in character and are considered by the authors to resemble changes observed experimentally in chronic acidosis rather than in hyperparathyroidism. In partially nephrectomized, renally insufficient rats main-

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tained on adequate diets, Pappenheimer (12) noted either no bone changes or only trifling ones.

These findings may be interpreted as indicating that in the usual case of adult chronic nephritis with renal insufficiency any increased requirement for calcium, if present, is supplied by the diet. For when Pappenheimer restricted the calcium intake of young partially nephrectomized rats severe skeletal lesions developed. Further, in the growing child when calcium requirements are high, long-standing renal insufficiency frequently leads to stunted and deformed skeletal growth.

All these observations suggest that the amount of calcium consumed in the diet is a factor in the development of bone lesions in renal insufficiency. If, as Richter and Eckert (1) have shown for parathyroidectomized rats, there is a natural tendency to increase calcium intake as needed, then a similar increase of calcium intake should be demonstrable in experimentally produced renal insufficiency, assuming, as the evidence already presented suggests, that an increased calcium requirement exists. The purpose of the experiments recorded here is to discover by the method of Richter (1) whether or not this is true.

METHODS

The procedures outlined by Richter and Eckert (1) were followed in most particulars. The rats were kept in individual cages to which were attached two 100 cc. graduated water bottles containing the required solutions and the daily intake from each was measured to the nearest cubic centimeter. The solutions used in the experiments in different combinations were: calcium lactate, U.S.P. 2.4%; sodium (dibasic) phosphate, U.S.P. 4.0%; distilled water. The animals were maintained on a constant low calcium diet, This consisted of the McCollum diet without the addition of calcium carbonate. Each animal was furnished with 40 gm. of food daily. The daily food intake was fairly constant and averaged about 20 gm.

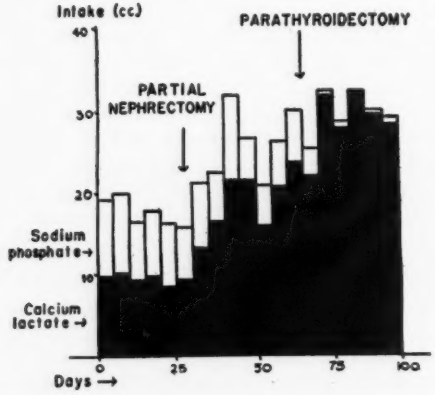
After a preliminary period of 25 days or more, during which the voluntary food intake of the intact animal was established, renal insufficiency was produced. Approximately two-thirds of the left kidney was excised and at a second operation from 7 to 10 days later the entire right kidney was removed. The animals were then continued on a measured fluid intake for at least 25 days postoperatively. In certain experiments complete extirpation of both parathyroid glands was performed in order to compare the results of this procedure with those of nephrectomy.

RESULTS

Voluntary intake of calcium lactate and sodium phosphate before and after partial nephrectomy and complete parathyroidectomy. Six albino rats were given a choice of 2.4% calcium lactate or 4.0% sodium phosphate but no water. The animals were litter mates and about three months of age. The average daily calcium intake for the 25-day preoperative period varied from 6.2 to 11.3 cc. (table 1) and the mean for all 6 animals was 9.5 cc. After partial nephrectomy the daily calcium intake varied from 12.5 to 24.1 cc. with a mean of 17.8 cc. There is thus shown a strikingly increased demand for the calcium solution which was constant in all 5 surviving animals (one died during the operative procedure) and approximated 100%.

The daily intake of sodium phosphate (table 1) during the preoperative period ranged from 4.4 to 8.3 cc. and the mean of all 6 rats was 5.9 cc. It is thus seen that rats, given a choice of only calcium lactate and sodium phosphate, drink both solutions but have a slight preference for the former. After partial nephrectomy, the phosphate intake remained essentially unchanged declining slightly in 3 animals and increasing slightly in the other 2 survivors. The mean for all 5 (6.0 cc.) was, however, almost exactly the same as in the preoperative period (5.9 cc.).

Complete parathyroidectomy (as verified by histological examination of the excised tissue) was performed at the end of the first postoperative period in order to see if a further alteration in calcium and phosphate intake would result. The rats stood



CALCIUM AND PHOSPHATE INTAKE OF *rat* 80 IN CONSECUTIVE 5 DAY PERIODS.

this operative procedure well and unlike non-nephrectomized rats showed no evidence of tetany. This was probably due to the protection afforded by the already augmented calcium intake. During the ensuing 30 days, the amount of calcium lactate consumed was again modestly increased. The mean value was 4.9 cc. per day more than before parathyroidectomy. More striking, however, was the sharp decrease in sodium phosphate consumption in all 5 animals to a mean value of only 1.5 cc. per day. The significance and consistency of these changes is best illustrated graphically (fig. 1) from the record of a single animal where the fluid consumption for 5-day intervals is represented.

A second group of 5 rats, also 3 months of age and litter mates was placed on a fluid intake of either 2.4% calcium lactate or 4.0% sodium phosphate. At the end of 25 days parathyroidectomy was performed. Within 24 to 48 hours after operation 4 of the 5 rats showed signs of tetany and one of these died. The other 3 began to con-

TABLE 1. VOLUNTARY INTAKE OF CALCIUM LACTATE AND SODIUM PHOSPHATE IN RATS AFTER PARTIAL NEPHRECTOMY AND PARATHYROIDECTOMY

Animal	Sex	Preoperative 1–25 days		After nephrectomy 35–60 days		After parathyroidectomy 61–90 days	
		2.4% calcium lactate	4.0% sodium phosphate	2.4% calcium lactate	4.0% sodium phosphate	2.4% calcium lactate	4.0% sodium phosphate
		cc./day	cc./day	cc./day	cc./day	cc./day	cc./day
80	M	10.8	5.7	20.4	4.7	23.6	1.6
81	M	8.7	4.8	died		—	
82	M	10.0	4.6	14.4	7.2	18.2	1.6
83	F	6.2	7.7	12.5	8.3	17.5	0.8
84	F	11.3	4.4	24.1	3.1	25.9	2.5
85	F	10.2	8.3	17.8	6.7	28.4	1.1
Mean		9.5	5.9	17.8	6.0	22.7	1.5

TABLE 2. THE VOLUNTARY INTAKE OF CALCIUM LACTATE AND SODIUM PHOSPHATE IN RATS BEFORE AND AFTER PARATHYROIDECTOMY

Animal	Sex	Preoperative 1–40 days		After parathyroidectomy 41–80 days	
		2.4% calcium lactate	4.0% sodium phosphate	2.4% calcium lactate	4.0% sodium phosphate
		cc./day	cc./day	cc./day	cc./day
90	F	8.7	6.5	16.6	1.6
91	F	7.7	8.4	14.3	1.3
92	F	6.8	5.8	4.4[1]	4.4[1]
93	M	10.8	6.8	died	
94	M	9.3	12.0	16.4	3.1
Mean		8.7	7.9	15.8	2.0

[1] Only one parathyroid gland was successfully excised. After operation this animal developed a neck infection and became badly nourished. This figure is excluded from the mean values.

sume increased quantities of calcium lactate and recovered. Their phosphorus intake was greatly diminished. This corroborates Richter and Eckert's findings. In the fifth animal (93) the operative field was obscured by hemorrhage and histological examination of the excised tissue revealed only one parathyroid gland. This animal failed to show an increased calcium or decreased phosphate intake. The data from these animals are presented in table 2.

These experiments indicate that either partial nephrectomy or complete parathyroidectomy cause an increased consumption of calcium lactate in rats given a choice between this solution and sodium phosphate. The chief difference in the results obtained from both procedures is that parathyroidectomy results in a decreased demand for sodium phosphate whereas the nephrectomized animals take as much of this solution as before operation.

Voluntary intake of calcium lactate or water and of sodium phosphate or water in partially nephrectomized rats. Since the rats in the above experiments had no choice of any indifferent fluid such as water it is not established whether the increased consumption of calcium lactate in the partially nephrectomized animals was due to a specific hunger for calcium or simply to increased thirst. In order to solve this question, 6 adult male rats weighing from 250 to 300 gm. were given a choice of either

TABLE 3. THE VOLUNTARY INTAKE OF CALCIUM LACTATE AND DISTILLED WATER IN PARTIALLY NEPHRECTOMIZED RATS

Animal	Preoperative 1–25 days		After nephrectomy			
			31–55 days		56–80 days	
	2.4% calcium lactate	water	2.4% calcium lactate	water	2.4% calcium lactate	4.0% sodium phosphate
	cc./day	cc./day	cc./day	cc./day	cc./day	cc./day
70	3.9	14.8	1.8	30.4	19.4	9.9
71	4.0	37.8	6.4	54.9	39.2	5.0
73	1.2	24.0	2.4	47.7	30.0	5.4
74	10.2	7.4	6.2	25.4	23.0	5.3
77	4.0	10.2	5.2	14.1	—	—
78	5.4	8.4	6.5	13.2	—	—
Mean	4.8	17.1	4.8	31.0	27.9	6.4

2.4% calcium lactate or distilled water. In the preliminary 25-day period they showed a preference for water over the calcium solution in all but one instance (table 3). After partial nephrectomy all the rats showed a markedly increased water consumption but the calcium lactate intake was essentially unchanged. In order to check the results of earlier experiments, 25 days after the second kidney operation the water was removed from the cages of 4 rats and replaced by 4.0% sodium phosphate. The rats continued to show a large total fluid intake but now took the bulk of their fluid in the form of calcium lactate.

These findings would seem to indicate clearly that the primary change after partial nephrectomy is a desire for more fluid which is met by increased water consumption if this fluid is available. If only calcium lactate and sodium phosphate are available the increased thirst is slaked by consumption of the calcium lactate not because of

TABLE 4. THE VOLUNTARY INTAKE OF SODIUM PHOSPHATE AND DISTILLED WATER IN PARTIALLY NEPHRECTOMIZED RATS

Animal	Preoperative 1–25 days		After Partial nephrectomy 31–60 days	
	4.0% sodium phosphate	water	4.0% sodium phosphate	water
	cc./day	cc./day	cc./day	cc./day
62	6.3	8.6	3.9	13.7
63	5.9	10.2	2.1	21.7
67	5.9	19.1	2.0	18.8
69	7.2	7.4	1.9	17.7
Mean	6.3	11.3	2.5	18.0

any increased hunger for calcium but because this solution is less distasteful than the phosphate one.

Rats which are kept on the low calcium diet used in these experiments and are given a choice of only 4.0% sodium phosphate or distilled water lose weight, become dehydrated and often succumb to partial nephrectomy. Of 10 animals in this group only 4 survived partial nephrectomy for longer than 30 days. The data from these are presented in table 4. Previous to operation the rats showed a preference for water over sodium phosphate. After operation, although the total fluid consumption was not greatly increased, the water intake was definitely greater and the phosphate intake correspondingly reduced.

DISCUSSION

The major premise on which this study was based is not supported by these experiments. The data fail to reveal any increased desire for calcium in partially nephrectomized rats as measured by the voluntary fluid intake and such as occurs after complete parathyroidectomy. On the other hand, there is demonstrable a decreased appetite for phosphate solutions in animals which had water available. If calcium was available the rats preferred this to phosphorus but the intake of the latter was maintained at the preoperative level. The explanation for this phenomenon may lie in the fact that with sufficient calcium unavailable in either fluid or solid elements of the diet the intake of phosphorus at even the prenephrectomy level would tend to aggravate the tendency toward phosphorus retention and this solution is avoided. When, however, the nephrectomized rat is consuming calcium in sufficient amounts to permit the excretion of phosphorus as calcium phosphate by way of the intestine,

this tendency for phosphate retention is overcome and the rat takes as much of this material as before operation.

As suggested by Richter and Eckert (1, 2) the level of intake of calcium and phosphorus may depend directly upon their concentration in the blood. The changes in blood calcium and phosphorus in the milder stages of renal insufficiency are inconstant, although hyperphosphatemia (4) and hypocalcemia (5) have been described. Of the two, the former is more frequently demonstrable. Recently Foweather and Pyrah (16) in 29 cases of nephritis of all types found the blood calcium to vary from 8.7 to 12.0 mg. per 100 cc. These findings would indicate that the blood calcium does not vary greatly from normal in nephritis and is just as often elevated as reduced. The blood phosphate was not elevated in the 4 cases of chronic renal disease in which this determination was made. Chanutin and Ludewig (17) found the blood calcium of rats to be unchanged after partial nephrectomy. The failure of the rats in the experiments reported here to show augmented calcium intakes may have been due to the fact that the blood calcium was not depressed.

The degree of renal insufficiency produced by the ablation of kidney tissue in these experiments was not determined by measurements of kidney function. However, the method employed for this purpose is a well established one. At the time the animals were killed the mean total mass of remaining and regenerated renal tissue of all partially nephrectomized animals was 0.85 gm. and was as low as 0.51 gm. in one instance. This is considerably less than the weight of one normal rat's kidney. Histologically the residual renal tissue in all cases was not normal but showed varying degrees of atrophy, fibrosis and in some, diffuse tubular dilatation. In addition one out of every two of these damaged kidneys showed marked calcification of tubules. The incidence and severity of tubular calcification could not be correlated with the intake of calcium. In fact, it was just as intense in some animals which had been given only sodium phosphate and water as in some of those which had consumed large quantities of calcium lactate. It seems hardly likely that all of these animals escaped some degree of renal insufficiency. Yet there is no indication from the individual records that any behaved strikingly different from the others of the same group in regard to postoperative intake of calcium lactate or sodium phosphate.

In addition to the morphological evidence, the striking postoperative change in water balance suggests that impaired renal function was attained. The increased thirst of the partially nephrectomized rats is not surprising. It has long been known that polyuria may accompany the loss of urinary concentrating power in renal insufficiency both in man and experimental animals. This is attributed by Rehburg (18) to the increased nitrogen concentration of the glomerular filtrate and the consequent inadequate reabosorption of water in the tubules. In the face of this excessive loss of fluid through the kidney a compensatory polydipsia is necessary to prevent dehydration.

The results of parathyroidectomy confirm the observations of Richter and Eckert (1) inasmuch as the calcium intake was increased, phosphorus intake diminished. No attempt was made in our experiments to prove that this change was independent of alterations in total fluid consumption. Richter and Eckert (1), however, appear to have established that this is the case.

SUMMARY

Partially nephrectomized rats on low calcium diets, given a voluntary choice of drinking calcium lactate solution or water, meet their increased fluid requirements by consuming increased quantities of water while their calcium lactate intake remains unchanged.

When given a voluntary choice of sodium phosphate solution or water, similar rats fail to take sufficient fluid to prevent dehydration and show a markedly reduced phosphate intake.

When water is excluded and a choice of calcium lactate or sodium phosphate solutions is afforded to partially nephrectomized rats, calcium consumptions is increased, while phosphorus intake remains unchanged.

Completely parathyroidectomized rats on low calcium diets given a choice of calcium lactate or sodium phosphate solutions, show increased calcium and decreased phosphorus intakes.

Complete parathyroidectomy in previously partially nephrectomized rats causes a further slight increase in calcium lactate and sharp decrease in sodium phosphate consumption.

The implications of these findings as they relate to altered calcium and phosphorus metabolism in renal insufficiency and secondary hyperparathyroidism are discussed.

REFERENCES

1. Richter, C. P., and J. F. Eckert: *Endocrinology* 21: 50. 1937.
2. Richter, C. P., and J. F. Eckert: *Am. J. Med. Sc.* 198: 9. 1939.
3. Mitchell, A. G.: *Am. J. Dis. Child.* 40: 101, 345. 1930.
4. Greenwald, I.: *J. Biol. Chem.* 21: 29. 1915.
5. Mariott, W. McK., and J. Howland: *Arch. Int. Med.* 18: 708. 1916.
6. Donahue, W., C. Spingarn and A. M. Pappenheimer: *J. Exper. Med.* 66: 697. 1937.
7. Hubbard, R. S., and J. A. Wentworth: *Proc. Soc. Exper. Biol. & Med.* 18: 307. 1920–21.
8. Kluge, E.: *Arch. f. path. Anat.* 298: 406. 1936.
9. Castleman, B., and T. B. Mallory: *Am. J. Path.* 13: 553. 1937.
10. Pons, J. A., and A. M. Pappenheimer: *Puerto Rico. J. Pub. Health & Trop. Med.* 13: 115. 1937.
11. Jarret, W. A., H. L. Peters and A. M. Pappenheimer: *Proc. Soc. Exper. Biol. & Med.* 32: 1211. 1935.
12. Pappenheimer, A. M.: *J. Exper. Med.* 64: 965. 1936.
13. Anderson, W. A. D.: *Arch. Path.* 27: 753. 1939.
14. Pappenheimer, A. M., and S. L. Wilens: *Am. J. Path.* 11: 73. 1935.
15. Ginzler, A. M., and H. L. Jaffe: *Arch. Path.* 27: 798. 1939 (abstract).
16. Foweather, F. S., and L. N. Pyrah: *Proc. Roy. Soc. Med.* 31: 593. 1938.
17. Chanutin, A., and S. Ludewig: *Am. J. Physiol.* 129: 242. 1940.
18. Rehburg, P. B.: The kidney in health and disease. Edited by Berglund, Medes, Huber, Longcope and Richards. Lea & Febiger, Philadelphia, 1935, p. 88.

PINEALECTOMY IN SUCCESSIVE GENERATIONS OF RATS

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STUDIES on the effect of pinealectomy over successive generations of animals have been the natural outgrowth of the positive results of Rowntree, Clark, Steinberg, and Hanson (1, 2), obtained by the administration of pineal extract to successive generations of rats. These investigators reported that the administration of a picric acid extract of beef pineal bodies to successive generations of rats resulted in retardation in growth and acceleration in development which became more marked with each succeeding generation. This experiment was repeated with a hydrochloric acid extract of beef pineal bodies (3) with qualitatively the same results as in the first experiment, though the results were this time less marked.

The positive results obtained by the administration of pineal extracts to successive generations of rats brought up the question of whether or not opposite results would be obtained by the pinealectomy of successive generations. D'Amour and D'Amour (4) reported a preliminary test with a few animals. Pinealectomy was performed before the age of 48 hours. A distinct increase in the weight of the third and fourth generations of operated rats was noted. The weights reported were for the ages of 3 ½ to 8 months. However, the authors stated that because of the small number of animals used this effect could not definitely be attributed to pinealectomy. No significant differences were found in the rate of development as indicated by the time of hair appearance, eye opening, and time of sexual maturity.

Einhorn and Rowntree (3) carried out pinealectomy of parent rats at the ages of 6 to 21 days (average age, 10 days) for 6 successive generations and found no appreciable effect upon the weight, rate of development, or age of sexual maturity.

Although it is only recently that the effect of pinealectomy in successive generations has been studied, the effect on individual animals has for many years been studied by different workers with inconsistent results. The outcome of these experiments is well summed up by Anderson and Wolf (5), who published a review of pinealectomy experiments in addition to their own studies on the subject. From their review of the literature Anderson and Wolf found that about half of the workers had reported an increase in the rate of growth and development from pinealectomy and that the others had reported entirely negative results. From their own experiments Anderson and Wolf concluded that complete removal of the pineal body of the rat between the ages of 1 to 3 days had no influence on the rate of growth, age of puberty, or the weight of the pituitary, thyroid, adrenal, or thymus glands of either sex and that it did not affect the weight of the testis in the male or the occurrence of normal estrous cycles in the female.

The experiment described in the present paper deals with pinealectomy in successive generations of rats. The negative results obtained confirm those of Einhorn and Rowntree, whose paper appeared during the progress of these experiments.

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MATERIALS AND METHODS

The animals used in this experiment were albino rats from an inbred colony of the Wistar strain.

The P_1 generation of test animals was made up of 5 pairs of rats, pinealectomized at the age of 20 to 21 days. Each pair was kept in a separate cage. Use was made of only those rats which had apparently been completely pinealectomized as shown by histological sections of the tissues removed. Completeness of removal was later checked by serial sections of the operative site in the P_1 and the succeeding generations of pinealectomized rats, and only the offspring of completely pinealectomized rats are included in the data.

For each P_1 pinealectomized rat a control from the same litter and of the same sex and approximately the same size and weight was selected. All the parent control rats were subjected to a control operation in which the pineal body was exposed but not removed.

The offspring of the P_1 generation are designated as the F_1 generation. In the F_1 generation of pinealectomized rats there were 106 animals and 6 pairs of these were pinealectomized and used as parents of the F_2 generation. From the F_1 controls 3 pairs of rats were given control operations and became the parents of the F_2 controls.

There were 149 rats born in the F_2 generation of pinealectomized rats, and of these 2 pairs of completely pinealectomized rats were used as parents of the F_3 generation, in which there were 24 rats, Two pairs of operated controls became the parents of the F_3 generations of control rats.

For parent rats there were selected animals of average size for their generation. Each litter was limited at birth to 8 rats. Runts and infected rats were not included in the data. Control and test animals were kept in the same room and given the same diet.

TABLE 1. THE EFFECT OF PINEALECTOMY OF SUCCESSIVE GENERATIONS OF PARENT RATS ON THE WEIGHT OF THE OFFSPRING

Age	Controls	F_1	F_2	F_3
days	*gm.*	*gm.*	*gm.*	*gm.*
0	5.1[1] (97) 4–6.5	5.1[1] (106) 3.5–6.2	5.2[1] (149) 4.3–6.3	5.1[1] (24) 4.6–6.1
7	10.1 (80) 7–14	9.9 (68) 7.5–12.5	12.4 (22) 8.8–15	9.7 (23) 7.7–11.8
14	17.9 (61) 10–30.9	17.9 (53) 10.1–23.7	14.7 (4) 12.6–16.2	16.5 (22) 13–22.2
21	24.8 (51) 16.1–38.4	27.4 (41) 13.4–34	29.0 (24) 19.5–39.3	24 (19) 20.5–31.4
30	45.8 (34) 30.5–48.8	50.3 (32) 36.8–59	51.9 (21) 28.8–74	40.4 (16) 25.6–52.5
40	69.5 (45) 52–92.8	73.7 (28) 51.3–95.6	76.5 (15) 66.2–94.5	63.3 (11) 46.6–81.4
50	89.1 (27) 64.3–140.3	90.3 (33) 73.7–129	103.6 (15) 81.6–121.8	89.6 (11) 72.0–113.3
60	108.9 (31) 75–141.9	133.2 (17) 100.3–173.6	134.2 (13) 120–154.9	108.2 (11) 84.4–131

[1] Under each average weight the range in weights is given. ( )=No. of Rats.

The rats were weighed and measured at birth and once a week up to the age of 21 days and thereafter every 10 days up to the age of 60 days. The time of hair appearance, tooth eruption, eye opening, and vagina opening were recorded for each rat. The rats were measured with vernier calipers from the tip of the nose to the base of the tail, and during this procedure were always held in the same position. Repetition of measurements on the same rat and same day showed this method to give results that varied within a range of 2 mm. The date of tooth eruption was taken as the time at which the incisors first appeared. The time of eye opening was recorded as the date at which both eyes were completely open. At first a record of the testis descent was kept, but this determination was found to vary so much that it was discontinued.

All pinealectomies were performed at the age of 20 to 21 days under ether anesthesia. A skin incision was made about ½ inch long in the midline with the midpoint of the incision over the parieto-occipital suture. A transverse incision was then made with a sharp scalpel through the occipital bone, and from each end of this incision another one was carried forward through the parietal bone. The bone flap thus formed was lifted forward without injury to the dura mater and its contained sinuses. A single slit was then made in the dura mater behind the junction of the sagittal and the transverse sinuses. Through the slit in the dura the points of a small pair of curved forceps were inserted until they were just anterior to the junction of the venous sinuses. The forceps were then closed and the pineal body removed. Usually the pineal body was removed blindly, but in some cases it could be seen through the slit in the dura mater before removal.

RESULTS AND DISCUSSION

The data are summarized in the accompanying tables. Table 1 gives the average weight, range of weights, and the number of rats upon which each figure is based for

TABLE 2. THE EFFECT OF PINEALECTOMY OF SUCCESSIVE GENERATIONS OF PARENT RATS ON THE LENGTH OF THE OFFSPRING

Age	Controls	F_1	F_2	F_3
days	*mm.*	*mm.*	*mm.*	*mm.*
0	48[1] (97) 43–52	48[1] (106) 44–55	49[1] (149) 45–55	48[1] (24) 43–50
7	63 (80) 54–77	63 (68) 54–70	67 (22) 62–74	65 (23) 63–70
14	79 (61) 69–100	79 (53) 68–90	77 (4) 73–82	84 (22) 78–90
21	98 (51) 80–113	101 (41) 81–112	104 (24) 85–120	100 (19) 97–113
30	119 (34) 105–139	126 (32) 109–136	128 (21) 103–143	130 (16) 101–133
40	140 (45) 125–164	144 (25) 128–157	141 (15) 143–158	140 (11) 130–155
50	153 (27) 137–170	160 (33) 143–174	162 (15) 146–170	153 (11) 140–168
60	165 (31) 152–180	176 (17) 160–206	175 (13) 174–180	167 (11) 152–180

[1] Under each average length the range in lengths is given. ( )=No. of Rats.

TABLE 3. THE EFFECT OF PINEALECTOMY OF SUCCESSIVE GENERATIONS OF PARENT RATS ON THE RATE OF DEVELOPMENT OF THE OFFSPRING

	Hair appeared	Teeth erupted	Eyes opened	Vagina opened
Controls	6 days (80)[1]	9–10 days (70)[1]	17 days (49)[1]	50.6 days (Av.) 40–57 (range) (29)[1]
F_1 Pin.	6 days (68)	9–10 days (60)	17 days (45)	50.2 days (Av.) 40–60 (range) (29)
F_2 Pin.	6 days (30)	9–10 days (24)	17 days (24)	44.2 days (Av.) 40–49 (range) (8)
F_3 Pin.	6 days (23)	9–10 days (23)	17 days (20)	57.3 days (Av.) 50–60 (range) (3)

[1] ( )=No. of Rats.

the controls and for the F_1 to F_3 generations of pinealectomized animals. Table 2 is similar to table 1 but deals with lengths instead of weights. Table 3 records the average age of hair appearance, tooth eruption, eye opening, and vagina opening and the number of rats upon which each figure is based.

Controls. There were 97 rats born in the control group, all the offspring of rats which had undergone control operations. Of these 31 lived to the age of 60 days. The death rate was much higher among control rats born in the summer, but yet those born in the summer did not differ appreciably in weight or rate of development from the control rats born at other times of the year.

Offspring of pinealectomized rats. There were 106 rats born in the F_1 generation, and of these 17 lived to the age of 60 days. No differences were noted between these rats and the control rats in general appearance and behavior. The average weight, length, and rate of development of the F_1 rats compare very closely to those for the controls, with the exception of weight at the age of 60 days. Here the average weight was 133.2 gm. for the F_1 pinealectomized rats as compared to 108.9 gm. for the controls. However, 60 days is the age at which there was the smallest number of rats. Also, the variation in weight among the same group of animals became greater as the age increased.

In the F_2 generation there were 149 rats born, of which only 13 reached the age of 60 days. This high death rate is probably accounted for at least in part by the fact that most of this generation were born in the summer months. The weights and lengths of the F_2 rats compare closely with those of the control group below the age of 50 days. At the ages of 50 and 60 days a greater difference occurred. But, as with the F_1 generation, this difference is not of great significance as it was at the older ages that there were the smallest numbers of rats and that the greatest variation would have normally occurred. The dates of hair appearance, tooth eruption, and eye opening were the same as for the controls. But the average age of vagina opening was somewhat earlier: controls, 50.6 days; F_2, 44.2 days. However, the earliest age of vagina opening was the same for both controls and the F_2 generation.

Twenty-four F_3 rats were born and 11 reached the age of 60 days. The average weights and lengths of the F_3 rats corresponded closely to those of the controls at the ages recorded, including the ages of 50 to 60 days. The F_2 rats were definitely heavier, on the average, at the age of 60 days than the controls and slightly heavier

than the F_1 rats. But the F_3 rats at the age of 60 days showed almost the same average weight as the controls: controls, 108.9 gm.; F_3, 108.2 gm. The ages of hair appearance tooth eruption, and vagina opening showed no significant differences from the controls.

In examining the data for the three successive generations of pinealectomized rats, one can find no progressive change in weight, length, or rate of development.

Microscopic examination of the endocrine glands from controls and the 3 generations of pinealectomized animals showed no noticeable differences. The tissues examined were pituitary, pineal, thyroid, thymus, pancreas, suprarenal, ovaries and testis.

The average weight and size at birth of the different generations of pinealectomized rats compared very closely to the average weight and size of the control rats at the time of birth. Theoretically the effect of pinealectomy of parent rats might be expected to show up at birth, if at all, as any effect obtained would most likely be due to the deprivation of the developing embryo of the pineal secretion normally obtained from the mother. According to the above suggested mechanism, pinealectomy of the male parent would play no part in any effect produced in the offspring. For pineal treatment or pinealectomy of the male to affect the offspring the spermatazoa would have to be affected, which seems unlikely. However, in the thymus experiments of Einhorn and Rowntree (6) no effect was obtained unless both parents were thymectomized.

Among the factors that might explain why pinealectomy in successive generations did not produce appreciable results when treatment with pineal extract has been reported effective are the following: (*a*) in the preparation of the extract active chemical substances not originally in the pineal tissue may have been formed; (*b*) the quantity of active material injected with the extract may have produced a much greater concentration in the blood than that normally produced by the pineal body; and (*c*) when the pineal body is removed, some other structure may take over its function.

SUMMARY

All test animals were pinealectomized at the ages of 20 to 21 days.

The P_1 generation of test animals was made up of 5 pairs of rats and produced 106 offspring in the F_1 generation. Six pairs of these were used as parents of the F_2 generation and produced 149 offspring. Two pairs of these were used as parents of the F_3 generation and produced 24 offspring.

All parent control animals were subjected to a control operation, in which the pineal body was exposed, but not removed. There were 97 animals born in the control group.

Pinealectomy of 3 successive generations of parent rats at the ages of 20 to 21 days produced no appreciable effect upon the body weight, body length, the rate of development, or the histological structure of the endocrine glands of the offspring.

REFERENCES

1. Rowntree, L. G., J. H. Clark, A. Steinberg and A. M. Hanson: *Endocrinology* 20: 348. 1936.
2. Rowntree, L. G., J. H. Clark, A. Steinberg and A. M. Hanson: *J.A.M.A.* 106: 370. 1936.
3. Einhorn, N. H., and L. G. Rowntree: *Endocrinology* 24: 221. 1939.
4. D'Amour, M. C., and F. E. D'Amour: *Proc. Soc. Exper. Biol. & Med.* 37: 244. 1937.
5. Anderson, D. H., and A. Wolf: *J. Physiol.* 81: 49. 1934.
6. Einhorn, N. H., and L. G. Rowntree: *Endocrinology* 21: 659. 1937.

EFFECT OF STEROID AND PITUITARY HORMONES ON EXPERIMENTAL DIABETES MELLITUS OF FERRETS[1]

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THE ACTION of various anterior pituitary and adrenal cortical principles on carbohydrate metabolism has been extensively studied. Scattered reports of various types suggest that the sex steroids also affect carbohydrate metabolism. Studies of the actions of sex hormone injections have led to results which can only be said to be controversial, or of uncertain interpretation or significance (1–12). Sex differences in carbohydrate and ketone levels have been demonstrated (13), and considerable evidence indicates that the glycosuria of either experimental or clinical diabetes mellitus can be reduced by estrogens (14–19), but this has been questioned (20); androgens also have been reported to diminish glycosuria (19, 21, 22).

We report here the effects of various sex and adrenal cortical hormones on the diabetic symptoms of partially depancreatized ferrets. Since previous work of this type has not been done in the ferret, the response of this species to the diabetogenic action of crude anterior pituitary extract (APE) was also investigated. The ferret was used because previous work has indicated that it is more responsive than other species to the cortin-like action of progesterone (3). An abstract of this work has been published (23).

METHODS

Mature 6 to 12-month-old females, weighing 500 to 700 gm., were used. Animals receiving steroid hormones were ovariectomized. They were kept in individual metabolism cages on a diet consisting of fresh, ground, lean meat (64.7%) devoid of all visible fat, raw pancreas (32.4%), cod liver oil (0.8%), dried yeast (0.6%), and a Na salt mixture (1.5%). In addition, 30 cc. of fresh milk was given daily. The salt provoked a moderate diuresis and thus prevented the retention of sizable portions of the total 24-hour urine in the bladder during collecting periods.

Food was given once daily and was consumed normally within 8 hours. The amounts were adjusted to maintain body weight, and then kept constant for periods of several months. Blood samples were drawn from the tail just before the feeding periods and sugar determined by the Shaffer (24) copper-iodometric method, with blood filtrates prepared by the Somogyi (25) zinc sulfate procedure. Urine sugar was determined by the Benedict quantitative method; non-protein nitrogen by micro-Kjeldahl analysis. Urine acetone was estimated semi-quantitatively by means of the Rothera color test on variously diluted urine specimens. Normal animals never gave a positive response by this test. The estimations are tabulated by plus marks. Roughly, 1+ indicates 0.002 to 0.005 gm. acetone; 2+, 0.006 to 0.015 gm.; 3+, 0.016 to 0.04; 4+, 0.05 to 0.13; 5+, 0.14 to 0.35; 6+, 0.36 to 0.90.

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The average daily sugar output for about a week both before and after experimental treatment established control figures on each animal for each experiment. The "before" control period is the more significant one because an enhanced glycosuria may occasionally linger after the causal stimulus is withdrawn (26).

Crude APE was made from fresh beef pituitaries. The anterior lobes were dissected in a semi-frozen condition, ground in sand, diluted with saline, frozen overnight, and then thawed and the debris removed by centrifugation. The extract was kept frozen, without preservatives, at $-5°$ C., and was active for as long as three months. In attempting to induce diabetes in intact animals, we prepared an AP extract as nearly as possible according to the directions of Young (27).

RESULTS

Intact ferrets. (*a*) *Effects of APE* (Fig. 1). Five mature ferrets and 2 2-month-old animals were injected with APE (doses gradually increased to the equivalent of 1 gm. anterior lobe tissue daily) for 10 to 25 days. No glycosuria appeared. A mild acetonuria occurred in 3 cases. A glycemic increase of 40 to 50 mg.% was induced, perhaps not specifically due to diabetogenic factors (28). Control experiments indicated that it was not caused by posterior lobe contaminants. The ferret, then, resembles the species investigated by Young (27) which in the intact state are relatively refractory to the diabetogenic action of APE.

The treatment did, however, induce a remarkable stimulation of body growth associated with a water and nitrogen retention (cf. 29–31). The usual effect was a rise in water intake and fall in urine volume. On diets which normally maintained body weight with only slight increment, APE induced a 23% mean weight increase during the first week of treatment. An average of half this gain was lost within 5 days after treatment was stopped (fig. 1); the rest was retained.

(*b*) *Effects of partial pancreatectomy.* In 10 cases an average of 1,625 mg. of pancreatic tissue was removed and fragments of 100 to 200 mg. left in the region of the bile duct. Daily urine sugar of 0.5 to 8.0 gm. appeared in every case, generally persisting for the several months of observation. Decline of the glycosuria indicated pancreatic regeneration, and could be counteracted by a second operation. Blood sugars in such animals ranged from 150 to 350 mg.%, and averaged approximately 250 mg.%. The blood sugar of intact animals ranged from 60 to 80 mg.%. The renal threshold for glucose approximated 150 mg.%. Acetonuria was generally absent, but sometimes occurred sporadically in small amounts if the urine sugars were high. The glycosuria was reduced 60% by a 30% reduction in food intake and was almost abolished by a 24-hour fast. Even with constant conditions of diet and care, however, there were considerable excursions in the daily glycosuria (see control periods, fig. 2–9). Maintenance of weight and good health was not difficult in these animals.

Partially depancreatized ferrets. (*a*) *Effect of APE* (table 1, fig. 9). Eight ferrets were tested for responses to active batches of APE. The equivalent of about 0.5 gm. fresh tissue was given in 2 or 3 days. In all cases glycosuria, hyperglycemia, acetonuria, polydipsia, and polyuria appeared or were greatly intensified, as in other species. One lot of APE after 6 months was unable to elevate urine sugar, but was still ketogenic. Another batch, tested in 4 animals, was, for reasons not known, the only one of 9 lots that was ketogenic, but never diabetogenic, even in doses equivalent to 1 gm. fresh tissue daily.

(*b*) *Effect of adrenal cortical extract*[2] (table 1, fig. 8). Large doses of cortin pro-

[2] The endocrine products used here were generously supplied us as follows: desoxycorticosterone acetate (cortate) and testosterone propionate (oreton) by Dr. Erwin Schwenk, Schering Corporation; adrenal cortical extract (eschatin) by Dr. Oliver Kamm, Parke, Davis & Co.; the various estrogens and progesterone by Messrs. Ehrlich and Rosenberg, the Tremond Corporation.

TABLE 1. AVERAGE FIGURES ON RESPONSE OF PARTIALLY DEPANCREATIZED FERRETS TO VARIOUS SUBSTANCES

Treatment	Daily dose	No. of animals used	Duration of treatment	Daily urine sugar					Change in acetonuria during treatment
				Before treatment[3]	During treatment			After treatment	
					gm.	gm. change and range	% change[4]		
AP Extract	0.1–0.3 g.[1]	8	days 1–7	gm. 1.8	4.2	+2.4 (+1.8–+4.17)	+133	gm. 2.1	Increased markedly in all cases
Cortin[2]	12–15 cc.	4	2	1.2	4.47	+3.27 (+1.61–+4.5)	+273	2.8	Increased in all cases
Desoxycorticosterone acetate	5–10 mg.	8	7.4 (4–9)	3.86	3.82	−0.04 (−1.22–+1.47)	−1	3.76	Increased in 2 cases only—not significant
Progesterone	10–25 mg.	5	9.5 (6–12)	1.99	2.43	+0.44 (+0.03–+0.67)	+22	2.68	None
Estrogen	1–3 mg.	6	12.5 (9–16)	3.34	4.53	+1.19 (+0.17–+2.93)	+36 (+55)	3.62	Increased in all cases
Estrogen	0.105 mg.	1	10	2.87	5.73	+2.86	+99 (+179)	—	Increased
Testosterone propionate	15–20 mg.	4	9	4.29	5.48	+1.19 (+0.02–+192.)	+28 (+38)	5.05	Increased in 3 cases

[1] Equivalent of 0.1–0.3 gm. fresh anterior lobe tissue.
[2] Parke, Davis & Co.'s Eschatin.
[3] A period of at least 4 days, generally a week or more.
[4] The figures in parentheses in this column are obtained if the latent periods before responses began are eliminated.

duced a marked and prompt enhancement of the glycosuria and ketonuria in 4 of 5 cases. The exceptional animal was non-glycosuric at the time treatment was started, but she was responsive to APE.

(*c*) *Effect of desoxycorticosterone acetate* (table 1, fig. 6). Large doses of this substance failed to exert any significant effect on 8 diabetic ferrets. In preliminary short-term experiments (not included in the tables) there seemed to be a depression of the glycosuria, as reported for human cases (32). Further experiments, however, revealed that this effect was neither constant nor permanent.

(*d*) *Effect of progesterone* (table 1, fig. 5). Because of the several reports of effects of progesterone on carbohydrate metabolism, it appeared likely that this sex steroid, if any, would be diabetogenic. In 2 of 5 animals there was a slight augmentation of the glycosuria during the later stages of treatment, but this did not subside when treatment was withdrawn and was probably spontaneous. The other cases gave clearly negative results.

(*e*) *Effect of estrogens* (table 1, figs. 2–4). Various estrogens (estradiol-17-carbaethoxylate, estradiol benzoate, and a purified but non-crystsalline preparation from mare urine) were given to 7 diabetic ferrets. The dose ranged from 1 to 3 mg. daily or the equivalent thereof. This dose proved lethal in 1 case (cf. 33). In the 6 surviving cases the average over-all result was a 36% increase in sugar excretion during treatment. If the latent periods, before responses began, are eliminated from the averages, there was a 55% rise in glycosuria. One case only gave no urine sugar response. The rise in glycosuria was preceded in 2 instances by an initial depression. A sharper initial drop was seen in one animal treated for only 2 days and not included in the tables.

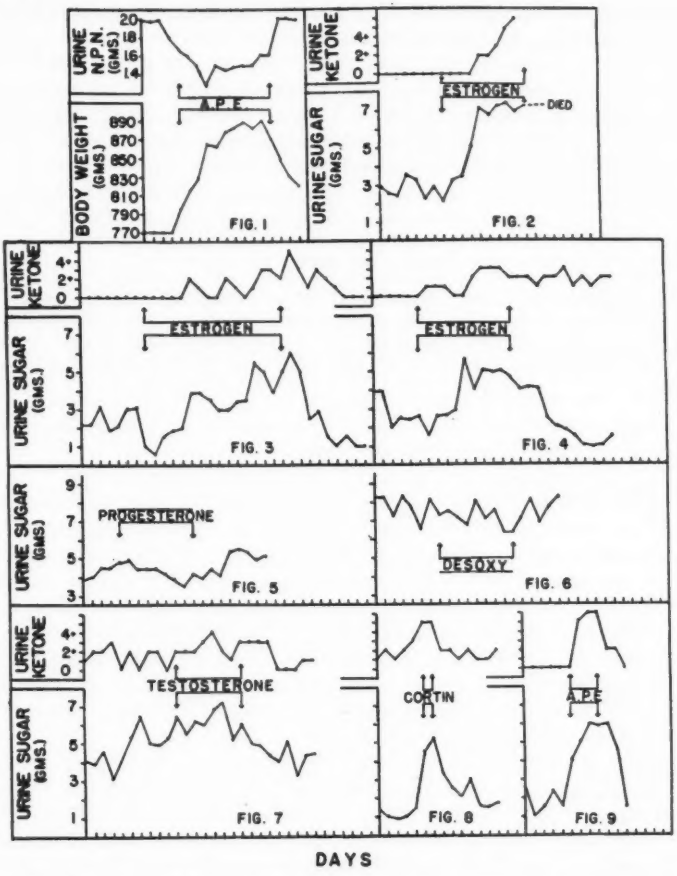

Fig. 1. Growth and N retention in APE treated intact ferret on constant diet.

Fig. 2–9. Response of individual representative partially depancreatized ferrets to various agents. Hormones were administered during intervals between the arrows. Where urine ketone curves are omitted, there was no detectable acetone throughout the experiment. Doses are indicated in the text and in table 1.

Response could not be correlated with dosage in the range used here, and increasing dosage during the experiment did not increase the sugar output. In 3 of the 5 responding cases the glycosuria fell to subnormal levels following treatment.

Acetonuria, completely absent during the "before" control period in 5 of the 6 animals, was produced or accentuated in every case at variable times during treatment.

An additional animal, given 0.105 mg. (1050 I.U.) E-17-carbaethoxylate daily for 10 days (a dose much smaller than that used in the above-mentioned cases) also exhibited a markedly accentuated diabetes (fig. 2), but death from unknown causes eliminated an "after" control period.

The customary weight losses which usually accompany estrogen treatment (in rats) did not occur. This observation is consistent with the view (34) that the weight-

reducing action of estrogen is largely due to its depression of appetite. The animals used here were on a constant daily diet.

(f) *Effect of testosterone propionate* (table 1, fig. 7). The mean effect was a 28% increase in urine sugar during the 9-day treatment period of 4 cases, or a 38% increase if latent periods are eliminated. This increase was entirely from 3 animals, since 1 gave no response. Acetonuria was likewise enhanced in the 3 responsive cases. Such responses resembled, in milder form, those with estrogen.

(g) *Blood sugar*. The blood sugar levels, measured in all the foregoing experiments, parallelled in general the urine sugar figures, and consequently are not detailed.

DISCUSSION

The results reported here on partial pancreatectomy and APE and cortin treatment in the ferret conform generally to those found in other species. Diabetes problems can be studied relatively easily and cheaply in this small-sized species. Total pancreatectomy is not difficult, as we determined in 2 cases, and the diabetic symptoms are severe. The partially operated animal is much more easily maintained in good health and was therefore used here. Like the partially depancreatized rat, it is highly responsive to known diabetogenic agents (35).

While this work was in progress, a number of publications on the effects of desoxycorticosterone in partially depancreatized rats (35, 36) and in related experiments (37–41) have indicated that our negative results were to have been expected. This hormone will, however, maintain carbohydrate levels in fasted, adrenalectomized rats (42) and restore them in adrenalectomized cats (43). We found no indications of a toxicity of desoxycorticosterone as seen in the dog (40).

Various reports in the literature as well as the biological and chemical relationship between the adrenal cortical and sex steroids suggest that the latter might affect carbohydrate metabolism. If such a common action exists, however, it should be exhibited by desoxycorticosterone, a substance which combines in several other respects both sex and cortin-like properties.

Progesterone, even in the huge dosage used here, had no appreciable effects, despite the fact that in previous experiments on intact ferrets we found it to elevate liver glycogen (3).

Previous work on estrogens has given rise to two types of results which are difficult to correlate: those in which these hormones seem to elevate blood sugar or liver glycogen (1, 2, 4, 8) and those in which they relieve diabetic symptoms (14–19). Estrogens have not been previously reported, as far as we are aware, to exaggerate symptoms of diabetes, although such results might be anticipated from the work of Janes and Nelson (2) and others. While the diabetogenic effects of estrogen which we observed were usually not of great magnitude, their consistency indicates significance—the more so because the experiments were carried out at intervals over a 9-month period. Whether this response is to be interpreted as a species peculiarity will have to be determined. Discussion of the mechanism involved would be premature. While estrogens cause adrenal enlargement, and hence might be diabetogenic as a result of adrenal stimulation, a test of this hypothesis would be difficult because of the high toxicity of estrogens after adrenalectomy (44, 45).

SUMMARY

Crude beef anterior pituitary extract did not produce glycosuria in intact ferrets but did cause a great stimulation of growth associated with water and nitrogen retention. Partial pancreatectomy in the ferret generally resulted in permanent glycosuria and hyperglycemia, and slight, if any, acetonuria. APE or cortin greatly

exacerbated the diabetes of partially depancreatized ferrets, as in other species. The following results were obtained with steroid hormones in partially depancreatized animals.

a), Desoxycorticosterone acetate and progesterone had no consistent effect.

b), Estrogen gave a rise in urine sugar in 6 of 7 cases, and a rise in acetonuria in all cases. On a constant diet, estrogen did not cause a weight loss.

c), Testosterone propionate had a slightly diabetogenic action.

ADDENDUM

Since completion of this manuscript Dr. Dwight J. Ingle, of the University of Pennsylvania, informed us of experiments conducted independently by him showing in a considerably more striking form the diabetogenic action of stilbestrol. This substance administered to partially depancreatized, non-glycosuric or mildy glycosuric rats on a constant medium carbohydrate diet produced an intense glycosuria in all of a large series of cases, and in normal, non-glycosuric rats on a constant high carbohydrate diet produced a mild glycosuria in the majority of a large series of cases. His results and those reported here on estrogens would seem to be mutually confirmatory. This work has been published in abstract (INGLE, D. J.: *Am. J. Med. Sci.* 201: 153. 1941.).

REFERENCES

1. GULICK, M., L. T. SAMUELS AND H. J. DEUEL, JR.: *J. Biol. Chem.* 105: 29. 1934.
2. JANES, R. G., AND W. O. NELSON: *Proc. Soc. Exper. Biol. & Med.* 43: 340. 1940.
3. GAUNT, R., J. W. REMINGTON AND A. EDELMANN: *Proc. Soc. Exper. Biol. & Med.* 41: 429. 1939.
4. ZUNZ, E., AND J. LABARRE: *Arch. Internat. Physiol.* 48: 287. 1939.
5. ZUNZ, E., AND J. LABARRE: *Arch. Internat. Physiol.* 48: 405. 1939.
6. ZUNZ, E., AND J. LABARRE: *Arch. Internat. Physiol.* 49: 464. 1939.
7. ENGELHART, E., AND O. RIML: *Klin. Wchnschr.* 13: 101. 1934.
8. COLLAZO, J. A., AND B. MARTI: *Ann. de med.* 38: 383. 1935.
9. FICHERA, G.: *Arch. Ital. di med. sper.* 4: 133. 1939.
10. RATHERY, F., AND J. TURIAF: *Compt. rend. Soc. de Biol.* 128: 155. 1938.
11. CORNIL, L., AND H. ROSANOFF: *Compt. rend. Soc. de Biol.* 129: 983. 1938.
12. GILDER, H., AND R. A. PHILLIPS: *Proc. Am. Physiol. Soc.*, p. 86. 1939.
13. DEUEL, H. J., JR., L. F. HALLMAN, S. MURRAY AND L. T. SAMUELS: *J. Biol. Chem.* 119: 607. 1937.
14. BARNES, B. O., J. F. REGAN AND W. O. NELSON: *J.A.M.A.* 101: 926. 1933.
15. NELSON, W. O., AND M. D. OVERHOLSER: *Endocrinology* 20: 473. 1936.
16. MAZER, C., D. R. MERANZE AND S. L. ISRAEL: *J.A.M.A.* 105: 257. 1935.
17. GESSLER, C. J., J. A. HALSTED AND R. P. STETSON: *J. Clin. Invest.* 18: 715. 1939.
18. SPIEGELMAN, A. R.: *Am. J. Med. Sci.* 200: 228. 1940.
19. BARTELHEIMER, H.: *Dtsch. Arch. f. klin. Med.* 184: 185. 1939.
20. COLLIP, J. B., H. SELYE AND A. NEUFELD: *Am. J. Physiol.* 119: 289. 1937.
21. LIPPROSE, O.: *Semana med.* 46: 911. 1939. (abst. *Endocrinology* 26: 558. 1940).
22. CORNIL, L., J. E. PAILLAS AND H. ROSANOFF: *Compt. rend. Soc. de Biol.* 129: 981. 1938.
23. GAUNT, R., G. DOLIN AND S. JOSEPH: *Anat. Rec.* (*Suppl.*) 78: 151. 1940.
24. SHAFFER, P. A., AND M. SOMOGYI: *J. Biol. Chem.* 100: 695. 1933.
25. SOMOGYI, M.: *J. Biol. Chem.* 86: 655. 1930.
26. GAEBLER, O. H., AND H. W. GALBRAITH: *Proc. Soc. Biol. Chem.*, p. 35. 1940.
27. YOUNG, F. G., *Biochem. J.* 32: 513. 1938.
28. RUSSELL, J.: *Physiol. Rev.* 18: 1. 1938.
29. TEEL, H. M., AND H. CUSHING: *Endocrinology* 14: 157. 1930.
30. GAEBLER, O. H.: *J. Exper. Med.* 57: 349. 1933.
31. LEE, M. O., AND N. K. SHAFFER: *J. Nutrition* 7: 337. 1934.
32. WINNETT, E. B., J. W. CALDWELL AND J. E. KAHLER: *J. Iowa State Med. Soc.* 30: 45. 1940.
33. SELYE, H.: *Am. J. Physiol.* 130: 358. 1940.
34. NELSON, W. O.: Personal communication.
35. LONG, C. N. H., B. KATZIN AND E. FRY: *Endocrinolgoy* 26: 309. 1940.
36. INGLE, D. J.: *Proc. Soc. Exper. Biol. & Med.* 44: 176. 1940.
37. GRATTAN, J. F., AND H. JENSEN: *J. Biol. Chem.* 135: 511. 1940.
38. LEWIS, R. A., D. KUHLMAN, C. DELBUE, G. F. KOEPF AND G. W. THORN: *Endocrinology* 27: 971. 1940.
39. SELYE, H., AND C. DOSNE: *Proc. Soc. Exper. Biol. & Med.* 44: 165. 1940.
40. KUHLMANN, D., C. RAGAN, J. W. FERREBEE, D. W. ATCHLEY AND R. F. LOEB: *Science* 90: 496. 1939.
41. WELLS, B. B.: *Proc. Staff Meet., Mayo Clinic* 15: 294. 1940.
42. HARRISON, H. E., AND H. C. HARRISON: *Proc. Soc. Exper. Biol. & Med.* 42: 506. 1939.
43. BRITTON, S. W., AND E. L. COREY: *Proc. Am. Physiol. Soc.* p. 20. 1940.
44. SELYE, H., C. M. HARLOW AND J. B. COLLIP: *Endokrinol.* 18: 81. 1936.
45. GAUNT, R., AND H. W. HAYS: *Am. J. Physiol.* 124: 767. 1938.

NOTES AND COMMENTS

ACTIVITIES OF THE FOOD AND DRUG ADMINISTRATION IN THE FIELD OF THE SEX HORMONES*

Estrogens. Commercial preparations of estrogenic materials are assayed by a modified Kahnt and Doisy technic.[1,2] The modifications consist of (*a*) the use of a standard and (*b*) designing the assay in such a way as to allow statistical evaluation of the results.

There are two standards devised by the Health Organization of the League of Nations[3,4] that have come into general use. The first of these international standards is a sample of crystalline estrone and the second is a sample of crystalline estradiol benzoate. The unit of each standard is defined in identical terms as "The specific oestrus-producing activity contained in 0.1 gamma of the standard preparation"[3,4] Although these definitions make no reference to the biological potency of the standards, the report of the committee which formulated them states "they wish to avoid any suggestion that these two units are equivalent in biological potency" (subhead 5, p. 623, reference 4).

The existence of two international standards with the correlated two international units, not equivalent in biological potency, has caused misunderatanding and confusion in the minds of many individuals. For example, the following statement has been published:[5] "Although I was also able to produce breast growth with injections of Ketohydroxyestrin (estrone), estradiol benzoate was, unit for unit, much more effective." The phrase "unit for unit" denotes that a comparison is made of the effectiveness of 1 I.U. of estrone with that of 1 I.U. of estradiol benzoate. Although these results confirm in man the difference in biological potency of the two standards which is generally recognized for rats, it would appear that this author regards an international unit as a single standard for estrogenic potency. The erroneous concept that an international unit of estrogenic potency is a single standard is widely accepted.

Each assay is designed to that in addition to determining the potency of the unknown the following information becomes available through statistical computations.[6]

1. Changes in the absolute sensitivity of a group of assay animals.
2. The standard error of the assay.
3. The significance of small differences in the responses to comparable doses of standard and unknown.
4. The capacity of animals to discriminate between graded doses.
5. The homogeneity of a group of animals used on any single assay.

During the four fiscal years July 1, 1936 to June 30, 1940, 243 samples of estrogenic preparations have been examined, of which 110, or 45.7%, failed to meet the requirements of the provisions of the Food and Drugs Act or its successor the Food, Drug and Cosmetic Act. It is gratifying to note, however, that the percentage of samples subject to such criticism has dropped from 64% in the year 1936–37 to 15% in the year 1939–40. Oil solutions that failed to meet the requirements of the law have decreased from 36.5% in 1936–37 to 5.2% in 1939–40. Legal action has been concluded against a number of oil solutions found to be

* This is a resume of a paper, the complete text of which appears in the April 1941 issue of *Journal of Clinical Endocrinology.*

[1] Allen, E., and E. A. Doisy: *J.A.M.A.* 81: 819. 1923.
[2] Kahnt, L. C., and E. A. Doisy: *Endocrinology* 12: 760. 1928.
[3] Dale, H. H.: *Quart. Bull. Health Organ. League of Nations.* Special Number. 4: 121. Jan. 1935.
[4] *Quart. Bull. Health Organ. League of Nations.* 4: 622. 1935.
[5] MacBryde, C. M.: *J.A.M.A.* 112: 1045. 1939.
[6] Miller, L. C., C. I. Bliss and H. A. Braun: *J. Am. Pharm. Assn.* 28: 644. 1939.

deficient in their labeled potency,[7] against several adulterated and/or misbranded aqueous preparations,[8] and against some products containing desiccated ovary in tablet, capsule or powder form based on representations made for them by the manufacturer or distributor.[9]

Progestins. The specific alteration of the endometrium produced by progesterone may be conveniently demonstrated using either the method of Allen[10] or Clauberg.[11] Examination of commercial preparations of oil solutions of progesterone has failed to reveal a single instance of material deviation from the claimed potency. On the other hand, not a single aqueous extract or a preparation of desiccated corpus luteum has been found to contain any demonstrable progestational activity.

Gonadotropins. Since there is no generally accepted preference for any of the methods described for the measurement of gonadotropic activity, it has been the practice of this laboratory to use the method described or referred to in the circular accompanying each sample. Only a small number of such samples has been examined but since more than half of them appear to fail to meet the requirements of the Act, the conclusion that more time could profitably be spent on the examination of these products is inescapable.

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WATER DISTRIBUTION IN THE BODY DURING THE PREGNANT AND THE PUERPERAL STATE

THAT the blood volume rises in pregnancy has been established in a number of studies on women[1,2,3,4] on sheep[5] and on rabbits.[6] This rise is thought to be especially great as parturition approaches, reaching 65% in the 9th lunar month.[4] During the postpartum period, the hemodilution and hypervolemia disappear in from 10 days to 2 weeks in women[4,7] and within a few days in the rabbit. It is generally agreed that the fall in blood volume is too great to be accounted for by hemorrhage during parturition.

The present investigation was undertaken principally to determine whether any organ or tissues served as a temporary reservoir during the early postpartum period until the body was able to get rid of the excess fluid produced by the hypervolemia of pregnancy.

Methods. Adult female guinea pigs were used, divided into 4 groups: control, early pregnant, late pregnant and early postpartum (72 hours or less). They were fed an adequate diet consisting of rabbit chow and cabbage. Food was withdrawn 24 hours before the animals were killed but water was left in the cage. Animals included in the "late pregnancy" group were practically at term when they were killed. The postpartum animals were separated from their young as soon as the birth was discovered (within 8 hours). The animals were weighed and then killed by a blow on the head and exsanguinated; the thyroids and adrenals were then removed. These organs were dissected free of extraneous tissue, washed

[7] *Notices of Judgment under the Food and Drugs Act.* 28325. 30221.
[8] *Notices of Judgment under the Food and Drugs Act.* 28698. 27268. 26973.
[9] *Notices of Judgment under the Food and Drugs Act.* 25561. 30964.
[10] ALLEN, W. M.: *Am. J. Physiol.* 92: 174. 1930.
[11] CLAUBERG, C.: *Zentralbl. f. Gynäk.* 54: 2757. 1930.
[1] ROWNTREE, L. G., G. E. BROWN AND G. M. ROTH: The Volume of the Blood in Health and Disease. W. B. Saunders, Philadelphia, 1929.
[2] GROLLMAN, A.: The Cardiac Output in Health and Disease. Charles C Thomas, Springfield, 1932, p. 256.
[3] DIECKMAN, W. J., AND C. R. WEGNER: *Arch. Int. Med.* 53: 71. 1934.
[4] COHEN, M. E., AND K. J. THOMSON: J.A.M.A. 112. 1556. 1939.
[5] BARCROFT, J., A. KENNEDY AND M. F. MASON: *J. Physiol.* 95: 159. 1939.
[6] SALVESEN, H. E.: *J. Biol. Chem.* 40: 109. 1919.
[7] KEITH, N. M., L. G. ROWNTREE AND J. T. GERAHTY: *Arch. Int. Med.* 16: 547. 1915.

momentarily in running water, blotted dry at once on filter paper and weighed in pairs to the nearest milligram. Samples of liver, skin and muscle were taken and the same procedure followed.

After the organs and tissue samples were weighed, the percentage of water was determined by placing them in a drying oven kept at a temperature of approximately 105° C. until a constant weight was reached.

Results. From the accompanying table it can be seen that there is no significant change in the water content of any tissue examined, either in early or late pregnancy or in the post-

WATER CONTENT OF TISSUES DURING PREGNANCY AND PUERPERIUM

Tissue	Normal		Early pregnancy		"p"[1]	Late pregnancy		"p"[1]	Postpartum		"p"[1]
	No. of animals	% Water	No. of animals	% Water		No. of animals	% Water		No. of animals	% Water	
Liver	31	74.1	6	74.2	.9	11	74.5	.9	11	74.3	.9
Muscle	24	78.9	6	79.1	.3	12	78.4	.4	10	78.5	.7
Skin	31	68.9	6	68.9	.8	13	69.1	.6	11	70.0	.6
Adrenals	31	68.2	6	70.4	.1	12	68.2	1.0	11	70.0	.05
Thyroid	30	74.7	6	76.7	.4	12	75.3	.9	11	74.3	.7

[1] "p" refers to probability of difference from non-pregnant animals occurring by chance. A significant difference should have a "p" of 0.05 or less.[8]

partum period, with the exception of the postpartum adrenal, which shows a slight (2.6%) but significant increase.

Discussion. It is reasonable to suppose that an excess of fluid in the blood stream is removed by the kidneys or that it is stored somewhere in the tissues. If the excess fluid of the blood in the postpartum period were taken up by the tissues, even if only temporarily, the tissues should show an increase in their water content, especially the skin and its underlying areolar tissue which is known to be a water depot. The results we obtained, however, clearly show that the water content of the skin, striated muscle, thyroid and liver remain unchanged during the postpartum period.

It is thought that the swelling of a gland when active is accompanied by increased water content of its cells. If this view is correct, the slightly increased water content of the adrenals suggests increased activity during the postpartum period although it is not clear why they should be more active at that time. It is obvious, however, that due to their small size they cannot be regarded as depots for water storage following the hypervolemia of pregnancy.

Since no important increase in the water content of the various tissues was found in the postpartum state, the indication is that the hypervolemia produced by pregnancy is probably reduced by diuresis and perhaps in the later stages by lactation. These points, however need further study.

Summary. There is no significant change in the water content of the skin, muscle, thyroid or liver of the normal adult guinea pig during early or late pregnancy or the postpartum period. There is a slight (2.6%) but statistically significant increase in water content of the adrenals during the postpartum period. The reduction of the hypervolemia of pregnancy to a normal blood volume shortly following parturition cannot be explained by water storage in the liver, thyroid, muscle or skin.

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[8] FISHER, R. A.: Statistical Methods for Research Workers., Oliver and Boyd, London, 4th Edition, 1932. p. 114 ff.

ORAL ESTROGENIC POTENCIES OF DIETHYLSTILBESTROL ETHERS

WE HAVE PREVIOUSLY REPORTED the oral estrogenic potencies of diethylstilbestrol and the natural estrogens.[1] With the object of showing the relationship of structure to activity several dialkyl ethers of diethylstilbestrol were synthesized and their activities determined.

The compounds dissolved in water or dilute alcohol were administered by stomach tube in 3 divided doses of 1 cc. during 24 hours. Spayed Wistar strain rats in groups of 20 or 30 animals were used in making the tests. Vaginal smears made 54, 60 and 70 hours after the initial dose were rated positive if they showed an essential disappearance of leucocytes replaced by squamous epithelial cells.

COMPARISON OF RELATIVE ESTROGENIC ACTIVITY OF DIETHYLSTILBESTROL AND ITS ETHERS

Compound	Total dose, γ	% in estrus
Diethylstilbestrol	3.0	60
Dimethyl ether	15*	60
Diethyl ether	30	60
Dipropyl ether	100	25

* Emmens[2] reported that the dimethyl ether was one-fifth as active as diethylstilbestrol when administered orally to mice.

Summary. The oral estrogenic potency of diethylstilbestrol diethers rapidly decreases as the length of the chain in the alkoxy group increases. These results may be in part explained by the lower water solubility of the higher members of the series and the consequent decrease in absorption of the compounds from the intestinal tract.

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THE PSYCHICAL ACTIVITY OF MALE AND FEMALE SEX HORMONES OF HORSE URINE

MANY INVESTIGATIONS of the antagonism between male and female sex hormones have been made, with a common finding of reciprocal inhibitory action between testosterone and estrone. It has been considered by many that the inhibition of sexual functions by the opposite sex hormone is due to the inhibition of the hypophysis and a secondary deficiency of gonadotropin.

Zondek[1] has shown the presence of a very high quantity of estrone in the urine and testes of the male horse. There is no doubt, however, that the behavior of the stallion shows a very intense male sexual instinct.

In order to study the question of any antagonism between the male and female sex hormones present in horse urine, we undertook the following experiments.

To study the influence of the male hormone, the "psychical test" of Steinach, Kun and

[1] SONDERN, C. W., AND J. L. SEALEY: *Endocrinology* 27: 670. 1940.
[2] EMMENS, C. W.: *J. Endocrinol.* 1: 144. 1939.
[1] ZONDEK, B.: Die Hormone des Ovariums und des Hypophysenvorderlappens, 2nd edition, Vienna 1936.

Peczenik[2] was used because a behavior test was desired, rather than a quantitative assay. The test consists in the restitution of the sexual instincts, interest for the female and coitus in castrated male rats (the animals lose these instincts within 4 weeks after castration). We used guinea pigs in these investigations instead of rats.

The test animals were 4 male guinea pigs castrated 3 months previously and tested at different times with females put in rut by injection of 1000 U of dimenformone (Organon). The castrated males showed no interest for these females. We also injected into these castrated males smaller amount of male sex hormones (1 mg. of testosterone propionate and once 2 cock comb units of erugon, Bayer) and did not obtain any reaction. This negative result shows that a relatively large amount of sex hormone is necessary for this test. Steinach's as well as our own former experiences indicate that one needs a quantity of 5 to 10 mg. of testosterone or 30 to 50 cock comb units. We used the urine of a 5-year-old stallion (mixed Arabian and "criollo" race), making a crude benzol extraction, without any separation of the male and female hormones. The extract (in olive oil solution) was injected on 4 successive days into the 4 castrate males as well as into a castrated female guinea pig. An amount corresponding to 80 cc. of horse urine was injected daily.

In the female there was observed all the changes in the vaginal smears characteristic for the presence of estrone (Allen and Doisy test) in rodents. On the fifth day, 3 of the male animals showed interest in the female and performed coitus repeatedly. The fourth castrate male did not show this last and most important reaction until the 6th day when he reacted in the same manner as the others. The pusillanimity, so characteristic of the behavior of the castrate in the presence of the female was lost.

Repetitions of this experiment gave the same result. The interesting conclusion is, that the same crude extract of horse urine produces the reactions of male, as well as of female sex hormones, and that apparently the presence of the opposite sex hormone does not inhibit the activity of the other.

A crude extract of the urine of a male horse produces estrus in the castrate female as well as a psychical reaction in the castrate male guinea pig. The presence of the contrary sex hormone does not inhibit the activity of the other one.

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[2] STEINACH, KUN AND PECZENIK: *Wien. klin. Wchnschr.* 49: 899. 1936.